Chemistry and biological activity of plants with traditional uses relevant to Alzheimer's disease

Howes, Melanie-Jayne Rosemarie

CHEMISTRY AND BIOLOGICAL ACTIVITY OF PLANTS WITH TRADITIONAL USES RELEVANT TO ALZHEIMER'S DISEASE

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MELANIE-JAYNE ROSEMARIE HOWES

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Department of Pharmacy

King's College London

Franklin-Wilkins Building

150 Stamford Street

London SE1 9RT

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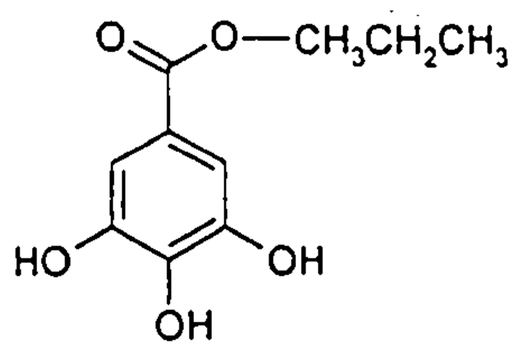
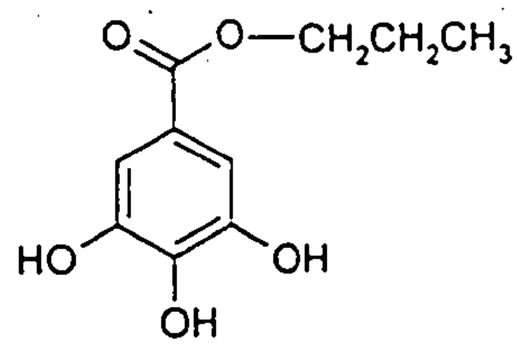
AMENDMENTS

Page	Line	Word/Structure	Amendment
14	29	matairesinal	matairesinol
78	5	formation AD.	formation in AD.
110	16	reported inhibit	reported to inhibit
121	7	has implicated	has been implicated
139	24	to a silica gel plates	to silica gel plates
169	18	psedomolecular	pseudomolecular
178	3	concIded	concluded
274	11	a second the hydroxyl substituent	a second hydroxyl substituent
282	5. 9	matairesinal	matairesinol
346	1	 Propyl gallate (**135**)	 Propyl gallate (**135**)
366	9	*Valariana*	*Valeriana*

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ABSTRACT

Investigations were conducted to determine the scientific basis for the traditional use of several plant species for dementia and other memory disorders, selected from Ayurvedic, Chinese and European herbal medicine, the plant parts investigated being used traditionally for central nervous system (CNS) disorders. Bioassays selected were associated with the pathological mechanisms that have consistently been associated with Alzheimer's disease (AD) and cognitive dysfunction.

A series of plant extracts and essential oils were assessed for *in vitro* anticholinesterase (antiChE) activity. Bioassay guided fractionation (including droplet counter-current chromatography (DCCC), flash column chromatography (FCC) and preparative thin layer chromatography (TLC)) of one of the most active antiChE extracts from *Convallaria majalis*, and the analysis of plant extracts and fractions using TLC and high performance liquid chromatography-mass spectroscopy (LC-MS), was conducted to identify the types of compound responsible for activity. Plant extracts, essential oils and essential oil components (identified using gas chromatography-mass spectroscopy (GC-MS)) were investigated for oestrogenic (and anti-oestrogenic) activity using a recombinant yeast screen; active extracts and essential oil constituents were also investigated in the human Ishikawa cell line, in receptor binding studies using the isolated α- and β-oestrogen receptors, and *in vivo*. Structure-activity relationships of essential oil constituents that demonstrated oestrogenic and anti-oestrogenic activity were also investigated using molecular graphics (with the computer program HyperChem™), to identify potential interactions with the oestrogen receptor, and were compared to known ligands for the oestrogen receptor.

A radioimmunoassay was conducted to assess inhibitory activity of plant extracts, essential oils and pure compounds against enzymes of the arachidonate cascade, to identify potential anti-inflammatory activity. Plant extracts and essential oils were also analysed in bioassays for inhibition of lipid peroxidation of bovine brain liposomes to determine anti-oxidant activity, for binding to $GABA_A$ and $GABA_B$ receptors, and for their effects on differentiation of neural stem cells isolated from rat brain and spinal cord.

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CONTENTS

ABBREVIATIONS

AA	arachidonic acid
ACh	acetylcholine
AChE	acetylcholinesterase
ACT	α-1-antichymotrypsin
AD	Alzheimer's disease
AGC	automatic gain control
ALA	alanine
AMPA	amino-3-hydroxy-5-methylisoxasole-4-propionic acid
ANOVA	analysis of variance
antiChE	anticholinesterase
APCI	atmospheric pressure chemical ionisation
ApoE	apolipoprotein E
APP	amyloid precursor protein
APR	acute-phase reactant
ARG	arginine
ATCh	acetylthiocholine
AU	absorbance units
BACE	beta-site APP cleaving enzyme
BBB	blood-brain barrier
BHT	butylated hydroxytoluene
BPH	benign prostatic hyperplasia
BSA	bovine serum albumin
Bu	butyl (C_4H_9)
BuChE	butylcholinesterase
Cdk5	cyclin-dependent kinase 5
C_6H_{14}	hexane
ChAT	choline acetyltransferase
$C_6H_5CH_3$	toluene
$CHCl_3$	chloroform
CH_3COCH_3	acetone
CH_3COOH	acetic acid

ChE	cholinesterase
C_2H_3N	acetonitrile
$(C_2H_5)_2O$	diethyl ether
CHOOH	formic acid
CID	collision induced dissociation
cm	centimetre
CNS	central nervous system
comp	composition
COX	cyclo-oxygenase
CPM	counts per minute
CPR	chlorophenol red
CPRG	chlorophenol red-β-D-galactopyranoside
CSF	cerebrospinal fluid
CSM	Committee on Safety of Medicines
CVF	cobra venom factor
d	density
Da	daltons
DARS	donkey anti-rabbit serum
DCCC	droplet counter-current chromatography
DCM	dichloromethane
DDT	DL-dithiothreitol
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulfoxide
DPBAE	diphenyl-boric-amino-ethyl-ester complex
DPPH	1, 1-diphenyl-2-picrylhydrazyl
DTNB	5, 5-dithiobis (2-nitrobenzoic acid)
E2	17β-oestradiol
EAV	extravascular albumin volume
EDTA	ethylenediamine tetra-acetic acid
EGTA	ethyleneglycol-tetra-acetic acid
ER	oestrogen (estrogen) receptor
ERα	alpha-oestrogen (estrogen) receptor
ERβ	beta-oestrogen (estrogen) receptor
ERE	oestrogen (estrogen) response element

ERT	oestrogen (estrogen) replacement therapy
EtOAc	ethyl acetate
EtOH	ethanol
eV	electron volts
F	fraction
FBS	foetal bovine serum
$FeCl_3$	ferric chloride
FGF	fibroblast growth factor
FID	flame ionisation detection
g	gram
GABA	γ-aminobutyric acid
GC	gas chromatography
GC-MS	gas chromatography-mass spectroscopy
Glc	glucose
GLU	glutamic acid
HAP	hydroxylapatite
HBSS	Hank's balanced salt solution
He	helium
hER	human oestrogen (estrogen) receptor
5-HETE	5-hydroxy-6, 8, 11, 14-eicosatetraenoic acid
HHT	17-hydroxyheptadecatrienoic acid
HIS	histidine
H_2O	water
H_2O_2	hydrogen peroxide
HOCl	hypochlorous acid
HPLC	high performance liquid chromatography
hr	hour
H_2SO_4	sulphuric acid
5-HT	5-hydroxytryptamine
HTAB	hexadecyltrimethylammonium bromide
IA	ibotenic acid
ICAM-1	intercellular adhesion molecule-1
ID	internal diameter
IL-1	interleukin-1

IL-6	interleukin-6
ITD	ion-trap detector
KOH	potassium hydroxide
KUT	Kami-utan-to
kV	kilovolts
L	litre
LBD	ligand binding domain
LC-MS	liquid chromatography-mass spectroscopy
LDL	low density lipoprotein
LEU	leucine
5-LOX	5-lipoxygenase
LTB_4	leukotriene B_4
LTP	long-term potentiation
m	metre
M	moles.L^{-1}
MAC	membrane attack complex
MAO	monoamine oxidase
MAP-2	microtubule-associated protein-2
MCA	Medicines Control Agency
MDA	malonaldehyde
Me	methyl (CH_3)
MeOH	methanol
µg	microgram
µM	micromolar
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
MPO	myeloperoxidase
MS	mass spectrometer or mass spectrum (dependent on context of text)
mw	molecular weight
m/z	mass/charge
N_2	nitrogen

NA	noradrenaline
$NaHCO_3$	sodium bicarbonate
Na_2HPO_4	disodium hydrogen phosphate
NaH_2PO_4	sodium dihydrogen phosphate
NDGA	nordihydroguaiaretic acid
NGF	nerve growth factor
NH_3	ammonia
nm	nanometre
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NPR	natural product reagent
NSAID	non-steroidal anti-inflammatory drug
nsb	non-specific binding
NSC	neural stem cell
PAF	platelet-activating factor
PBN	N-tert-butyl-α-phenylnitrone
PBS	phosphate buffered saline
PCB	polychlorinated biphenyl
PEG	polyethylene glycol
PGE_2	prostaglandin E_2
P+L	polyorninthine and laminin
PMN	polymorphonuclear leukocyte
***p*-NP**	*p*-nitrophenol
***p*-NPP**	*p*-nitrophenol phosphate
PpG	propyl gallate
$^{n}PrOH$	n-propanol
PS	petroleum spirit (40°-60°)
psi	pounds per square inch
PUFA	polyunsaturated fatty acid
RA	rosmarinic acid
RAL	raloxifene
R_f	retention factor
Rha	rhamnose

RIA	radioimmunoassay
R_t	retention time
SAM	senescence accelerated mice
SAP	serum amyloid P
s.c.	subcutaneous
SD	standard deviation
SDT	Shao-yin-ren shi-quang-da-bu-tang
SEM	standard error of the mean
SFM	serum free medium
SOD	superoxide dismutase
spp.	species
SSRI	selective serotonin reuptake inhibitor
t	time
TBA	thiobarbituric acid
TBS	Tris-buffered saline
TBS+	Tris-buffered saline containing 0.01% Triton X-100
TCM	traditional Chinese medicine
TIC	total ion current
TJM	traditional Japanese medicine
TLC	thin layer chromatography
TNF	tumour necrosis factor
TOR	torsion angle
TXB_2	thromboxane B_2
UK	United Kingdom
USA	United States of America
UV	ultraviolet
VIP	vasoactive intestinal peptide

LIST OF FIGURES

LIST OF TABLES

LIST OF STRUCTURES

30	alisol B	109, 349
31	alisol B monoacetate	109
32	alisol C	109
33	alisol C monoacetate	109
34	alismol	109
35	alismoxide	109
36	perlolyrine	112
37	3, 4, 5-trimethoxy-cinnamic acid	114
38	1, 2, 3, 7-tetramethoxyxanthone	114
39	sinapinic acid	115
40	tetrahydrocolumbamine	115
41	emodin	116
42	physcion	116
43	resveratrol	116
44	2, 3, 5, 4'-tetrahydroxystilbene 2-*O*-β-D-glucopyranoside	116
45	danshensu	120
46	convalloside	123
47	strophanthidin	189
48	cymarin	189
49	K-strophanthin-β	190
50	convallatoxin	190
51	bornyl acetate	192
52	myrcene	192
53	α-pinene	192
54	β-pinene	192
55	γ-terpinene	192
56	1, 8-cineole	194, 237
57	camphor	194, 237
58	borneol	194
59	gossypol	194
60	anaferine	197
61	cuscohygrine	197
62	tropine	197

63	hyoscine	197
64	hyoscyamine	197
65	sitoindoside IX	198
66	sitoindoside X	198
67	tenuifolin	230
68	citronellal	237
69	citronellol	237
70	geranial (citral a)	237, 289
71	neral (citral b)	237, 289
72	geraniol	238
73	(+)-limonene	238
74	linalool	238
75	nerol	238
76	ocimene	238
77	(+)-calarene	246
78	caryophyllene oxide	246
79	β-cedrene	246
80	(-)-cubebene	247
81	α-humulene	247
82	*trans*-caryophyllene	247
83	nerolidol	247
84	eugenol	248
85	6-methyl-5-hepten-2-one	248
86	nonanal	248
87	oestrone	278
88	sitosterol	282
89	genistein	282
90	coumestrol	282
91	matairesinal	282
92	zearalanol	283
93	nonyl phenol	283
94	hexachlorocyclohexane	283
95	raloxifene (RAL)	286
96	dihydroxybiphenyl	286

97	naphthol-2	286
98	butylparaben	286
99	anethole	287
100	retinaldehyde	289
101	asiaticoside	299
102	asiatic acid	300
103	gossypin	301
104	quercetin	301
105	gnaphalin	301
106	caffeic acid	302
107	chlorogenic acid	302
108	rosmarinic acid (RA)	303
109	withaferin A	305
110	prednisolone	305
111	jatrorrhizine	306
112	dauricine	306
113	cryogenine	307
114	luteolin	311
115	tanshinone I	313
116	tanshinone IIa	313
117	cryptotanshinone	313
118	neocryptotanshinone II	313
119	tanshindiol A	313
120	tanshindiol B	313
121	isotanshinone I	313
122	isotanshinone II	313
123	salvianolic acid A	314
124	salvianolic acid B	314
125	salvianolic acid C	314
126	solenolide A	315
127	solenolide E	315
128	myristicin	328
129	luteolin 3'-glucuronide	335

130	2-(3, 4-dihydroxyphenyl)-1, 3-benzodioxole-5-aldehyde	335
131	aspirin (acetyl salicylic acid)	341
132	salicylic acid	341
133	thiobarbituric acid (TBA)	342
134	malonaldehyde (MDA)	342
135	propyl gallate (PpG)	346
136	orientalol A	348
137	orientalol B	348
138	orientalol C	348
139	celastrol	349
140	3-(3, 4-dihydroxyphenyl) lactamide	354
141	2, 10, 11-trihydroxy-8-methoxy-1, 6, 7, 8-tetrahydro-2H-benzo[e]azecine-3, 5-dione	354
142	carnosol	355
143	carnosic acid	355
144	methyl carnosate	355
145	rosmanol	355
146	α-thujone	357
147	carvacrol	357
148	butylated hydroxytoluene (BHT)	361
149	spinosin	369
150	swertisin	369
151	euxanthone	382

CHAPTER 1

Introduction

1.1 Alzheimer's Disease

The word "dementia" comes from the Latin word *demens*, meaning to be out of one's mind. There are numerous causes of dementia, including Lewy body disease (Lewy bodies are composed of proteinaceous material, which cause neuronal damage), Pick's disease, cerebrovascular disease and Alzheimer's disease (AD), the last which is reported to be the most common form of dementia (Evans *et al.*, 1989; Nordberg, 1996). Alzheimer's disease is estimated to account for 50% - 60% of dementia cases in persons over 65 years of age (Francis *et al.*, 1999). Dementia may also be a feature of other conditions, including Parkinson's disease, Huntington's disease and Creutzfeld-Jacob disease (CJD). The varieties of conditions that may feature dementia make their diagnosis and subsequent treatment difficult.

AD is a progressive, neurodegenerative disease that primarily affects the elderly population, and is a major public health concern. It is estimated that approximately 800, 000 people in Britain and almost 4 million Americans have AD, and it is predicted that this figure may reach 9 million by 2004 (Gopinath, 1998; Max, 1993; Yankner and Mesulam, 1991); AD has been estimated to affect 10% of the population in the US over the age of 65 years (Evans *et al.*, 1989). It has been proposed that delaying the onset of AD may reduce the number of cases by up to 50% (Gopinath, 1998; Marx, 1996). Therefore, knowledge of the mechanisms that may initiate or accelerate AD may lead to the development of effective treatments. The costs of AD in the UK are reported to be over £1, 600 million each year, and close to US $100 billion each year in the US (Ernst and Hay, 1994; Gopinath, 1998), therefore effective treatments may not only alleviate AD symptoms and suffering, but may also reduce the economic burden to society.

There has been extensive research into the causes and pathogenesis of AD since it was first described by Alois Alzheimer in 1907. However, understanding of this complex disorder is still very limited, and a more complete understanding of AD is necessary

for drug discovery to proceed successfully, to identify a cure for this debilitating disease.

1.1.1 Pathology and Symptoms of Alzheimer's Disease

1.1.1.1 Symptoms Associated with Alzheimer's Disease

The main symptoms associated with AD involve cognitive dysfunction, primarily memory loss (Desgranges *et al.*, 1998; Förstl *et al.*, 1995; Grafman *et al.*, 1990; Grosse *et al.*, 1991). Language deficits, depression, behavioural problems including agitation, mood disturbances and psychosis are also features associated with the later stages of AD (Kumar *et al.*, 1998b; McGuffey, 1997; Wragg and Jeste, 1989). The neurobiological basis underlying memory impairment in AD is not fully understood. Although increasing age is a risk factor for AD development, AD may also occur at an earlier age, usually between ages 35 to 60 years (early onset AD). It is reported that there may be neuropathological and neurochemical differences between early and late onset AD cases (Rosser *et al.*, 1984; Zubenko *et al.*, 1989). Late onset AD is characterised by more profound impairment on semantic memory processing, which has been correlated with activity in the temporoparietal and frontal association cortices of the left hemisphere of the brain (Desgranges *et al.*, 1998; Grosse *et al.*, 1991).

1.1.1.2 Neurofibrillary Tangles and Amyloid Plaques

The pathological features of AD that occur, which are identified in the post-mortem brain, are neurofibrillary tangles, located in neuronal cytoplasm and composed of paired helical filaments containing abnormally hyperphosphorylated tau protein, and diffuse and neuritic plaques composed of degenerating neurites and proteins, which include synucleins (involved in synaptic membrane formation), apolipoprotein E (ApoE) and, primarily β-amyloid (Clayton and George, 1998; Crystal, 1993; Glenner and Wong, 1984; Goedert, 1999; Ihara *et al.*, 1986; Johnson and Jenkins, 1996; Lu *et al.*, 1999; Masters *et al.*, 1985; Ruben *et al.*, 1997; Wisniewski and Frangione, 1992). Because of abnormal hyperphosphorylation, tau from paired helical filaments cannot bind to microtubules or promote microassembly (Alonso *et al.*, 1994; Lu and Wood,

1993), which may lead to impaired neurotransmission. It has been reported that tau is phosphorylated by a complex formed between a protein (p25) and an enzyme (cyclin-dependent kinase 5 (Cdk5)); in AD higher levels of Cdk5 result in binding to p25, rather than only binding to p35, the precursor of p25, which promotes the subsequent hyperphosphorylation (Evans, 2001). The protease calpain converts p35 to p25 (Lee *et al.*, 2000; Nath *et al.*, 2000; Taniguchi *et al.*, 2001), so may promote neurofibrillary tangle formation. Inhibitors of calpain may prove useful in preventing neurofibrillary tangle formation.

Hyperphosphorylation of tau however, is not essential to the development of neurofibrillary pathology and oxidative stress may also be a contributing factor (Johnson and Jenkins, 1996). Neurofibrillary tangles interfere with intracellular transport, cell structure and contribute to neuronal cell death (Blass, 1993; Yankner and Mesulam, 1991). Neurofibrillary tangles and senile plaques were first described by Alois Alzheimer in 1907, following postmortem analysis of a patient with dementia (Blass, 1993; Johnson and Jenkins, 1996; Yankner and Mesulam, 1991). A correlation between the number of neurofibrillary tangles and cognitive impairment in Alzheimer's disease has been identified (Arriagada *et al.*, 1992; Roth *et al.*, 1967).

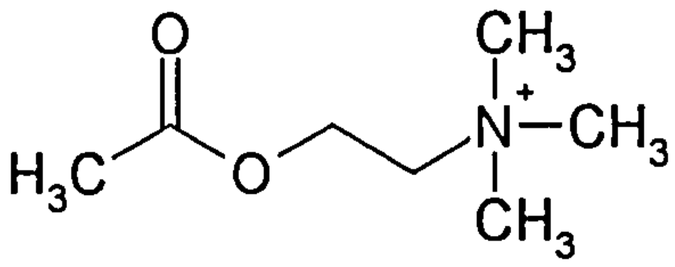
The formation of senile plaques may occur as a result of abnormal metabolism of amyloid precursor protein (APP). APP may occur as a transmembrane protein, or in a secreted form, which has been located in plasma and cerebrospinal fluid (Ghiso *et al.*, 1989; Podlisny *et al.*, 1990). The physiological role of APP is unclear, but has been reported to be required for the maintenance of neuronal and synaptic structure and function and cell adhesion (Huber *et al.*, 1997; Qui *et al.*, 1995; Saitoh and Mook-Jung, 1996). The secreted form of APP has been proposed to be involved in regulation of blood coagulation, wound-healing, neurite extension, cell adhesiveness and cell growth and differentiation (Araki *et al.*, 1991; Cole *et al.*, 1990; Cunningham *et al.*, 1991; Jin *et al.*, 1994; Milward *et al.*, 1992; Saitoh *et al.*, 1989; Schubert *et al.*, 1989; Smith *et al.*, 1990). The functions associated with APP may be linked with AD development. For example, secreted APP may participate in neuronal outgrowth, which may accelerate AD pathogenesis (Ihara, 1988), but further research is necessary to establish links between APP function and AD. APP is cleaved by proteases (e.g. β- and γ-secretases) to yield the abnormal insoluble form of β-amyloid (Chen, 1997); inhibition of these enzymes may be exploited for AD therapy.

It is unknown if the plaques are responsible for AD onset (i.e. they trigger neuronal degeneration), or if they are a feature of disease progression (i.e. they occur as a result of neuronal degeneration induced by other factors). β-Amyloid has been shown to induce neuronal toxicity *in vitro*, which may be explained by promoting neuronal oxidative stress, and by increasing susceptibility of neurons to excitotoxins (Behl *et al.*, 1992; Behl *et al.*, 1995; Behl *et al.*, 1997; Koh *et al.*, 1990; Mattson *et al.*, 1992; Yankner *et al.*, 1990). The exact mechanisms by which β-amyloid is neurotoxic remain to be established, but interactions with cell membranes and interference with Ca^{2+} transport and elevation of Ca^{2+} intracellular levels have been implicated in its neurotoxicity (Mattson *et al.*, 1992; Wu *et al.*, 1997). Fibrillar β-amyloid administration to aged rhesus monkey cerebral cortex resulted in tau phosphorylation, microglial proliferation and neuronal loss, but administration did not induce toxic effects in the young adult rhesus monkey, indicating that aging may render the brain vulnerable to β-amyloid toxicity (Geula *et al.*, 1998). This may reflect the loss of protective factors, or increased levels of neurotoxic co-factors, or perhaps impaired degradation of β-amyloid, with increasing age. Therefore, β-amyloid may be a significant factor in promoting neuronal degeneration and is a possible cause of AD onset in the elderly population.

It has been proposed that β-amyloid may occur as a result of neuronal degeneration as part of the disease process (Cummings *et al.*, 1996) so may play a significant role in disease progression, but not in disease onset. It may be that β-amyloid promotes neuronal degeneration in the early stages of AD pathogenesis, but is also generated as a result of disease progression, leading to further neuronal degeneration. It is also possible that an imbalance between the neurotrophic and neurotoxic functions of APP occur in AD pathology. It is apparent that the association of APP and β-amyloid with AD pathology is complex and further research is necessary to improve understanding of AD pathogenesis.

1.1.1.3 Alterations in Neurotransmitter Systems

Alterations in neurotransmitter systems, including acetylcholine (ACh) (**1**), dopamine, γ-aminobutyric acid (GABA) (**16**), glutamate, 5-hydroxytryptamine (5-HT) and noradrenaline (NA) have been associated with AD pathology (Advokat and Pellegrin, 1992; Fowler *et al.*, 1992; Giacobini, 1990; Nordberg, 1992; Palmer *et al.*, 1987a; Palmer *et al.*, 1987b; Perry *et al.*, 1978; Perry, 1986; Plotkin and Jarvik, 1986; Read, 1987; Reinikainen *et al.*, 1988; Seidl *et al.*, 2001; Storga *et al.*, 1996; Uchihara *et al.*, 1992).



Acetylcholine (1)

A consistent neuropathological occurrence associated with memory loss, is a cholinergic deficit, which has been correlated with the severity of AD (Bierer *et al.*, 1995; Collerton, 1986; Giacobini, 1990; Perry *et al.*, 1978; Perry, 1986; Plotkin and Jarvik, 1986; Read, 1987). This is more pronounced in the hippocampus and cerebral cortex, which are involved in cognitive function. Postmortem analysis of the brains of AD patients shows that the neurons of the basal nucleus of Meynert in the basal forebrain, a major source of cholinergic innervation of the cerebral cortex (responsible for higher learning function), undergo selective degeneration and atrophy (>75%) (Whitehouse *et al.*, 1982). The neurons of the nucleus basalis of Meynert are also reported to project to the amygdala, brainstem, hippocampus, entorhinal cortex and neocortex (Johnston *et al.*, 1979; Whitehouse *et al.*, 1982). The amygdala and basal forebrain form part of the limbic system, which mediates emotional behaviour. The degeneration of the neurons of the nucleus basalis of Meynert may therefore influence a number of processes involving cognition and behaviour.

Impairment of cholinergic neurotransmission has also been reported during normal aging, not associated with AD (de Lacalle *et al.*, 1991) but degeneration of cholinergic neurons is more pronounced in AD, identified by a reduction in cholinergic markers such as choline acetyltransferase (ChAT) activity, ACh (1), acetylcholinesterase (AChE) and muscarinic and nicotinic receptors (Bierer *et al.*, 1995; Bowen *et al.*, 1976; Court *et al.*, 2001; Davies, 1979; Davies and Maloney,

1976; London *et al.*, 1989; Martin-Ruiz *et al.*, 2000; Nordberg, 1993; Perry *et al.*, 1977a; Perry *et al.*, 1977b; Perry *et al.*, 1990; Rosser *et al.*, 1984; Shimohama *et al.*, 1986; Whitehouse and Kalaria, 1995). Cognitive impairment in patients with mild AD was comparable to anti-cholinergic treatment (using scopolamine) of control individuals (Christensen *et al.*, 1992), which correlates with the cholinergic deficit in AD.

The basis for this cholinergic abnormality is unknown. β-Amyloid infusion into rat brain has been shown to decrease ChAT activity in the frontal cortex and hippocampus, and also decreased ACh (1) and dopamine release from cholinergic and dopaminergic neurons respectively (Itoh *et al.*, 1996a; Nabeshima and Nitta, 1994; Nitta *et al.*, 1994). β-Amyloid plaque deposition may therefore contribute to the neurotransmitter abnormalities observed in AD. However, this does not explain the relatively selective loss of cholinergic neurons.

Cholinergic neurons use choline for two purposes: in the form of ACh (1) for neurotransmission and in the form of phosphatidylcholine, for cell membrane maintenance. It has been proposed that, in AD, choline metabolism is disturbed and under these conditions, cholinergic neurons may use membrane-bound choline for neurotransmission (Wurtman, 1992). This may result in impaired cell membrane structure and neuronal degeneration, and may also expose the transmembrane domain of APP to enzymes that metabolise APP to give β-amyloid, which may be deposited to form the senile plaques. Other theories that have been proposed to explain cholinergic impairment in AD include decreased energy metabolism, which may consequently affect APP metabolism, arising from oxidative damage to mitochondrial DNA (Gabuzda *et al.*, 1994; Mecocci *et al.*, 1994). However, impaired energy metabolism may also affect other neurons, and would perhaps not be selective for cholinergic neurons. Further knowledge regarding the relatively selective loss of cholinergic neurons in AD is necessary to validate the theories proposed, and identify potential causes of AD.

Decreased cholinergic activity in AD has also been associated with enhancement of APP processing, so perhaps increases senile plaque formation (Haas *et al.*, 1995). Some studies have shown that cholinergic abnormalities, including decreased cholinergic neurons in the basal nucleus of Meynert and decreased ChAT activity, were not observed in the brains of AD patients (Palmer *et al.*, 1986; Pearson *et al.*, 1983). As the diagnosis of AD is often difficult, due to symptomatic and pathological

similarities to other forms of dementia, it may be possible that the cases that showed no cholinergic impairment may not have been caused by AD. It may also be possible that AD may exist as several subtypes, which may vary in their pathology and severity of cholinergic abnormalities. This would make diagnosis and treatment more difficult.

1.1.2 Proposed Causes of Alzheimer's Disease

1.1.2.1 Genetics

Although increasing age appears to be the main risk factor for AD, identification of the cause or causes in the majority of cases of AD has yet to be established. Several factors have been proposed to cause AD, including genetics.

In a small proportion of AD cases, a gene on chromosome 21 has been identified, which initiates abnormal metabolism of β-amyloid to promote the formation of the insoluble form located in senile plaques; this rare cause of AD has been associated with early onset AD (Goate *et al.*, 1991; Heston *et al.*, 1981), but does not explain the majority of other cases. Patients with late onset AD have not shown linkage to chromosome 21 markers (St George Hyslop *et al.*, 1990). Chromosome 21 is responsible for Down's syndrome, and may explain why the brains of Down's syndrome patients show similar pathology to AD, and why relatives of patients with Down's syndrome also have an increased risk of developing AD (Griffin *et al.*, 1989; Masters *et al.*, 1985; Rumble *et al.*, 1989). . However, chromosome 21 does not explain all cases of early onset AD, and is perhaps an infrequent cause of early onset AD in the general population (van Duijn *et al.*, 1991).

Genes located on chromosomes 1 and 14, which code for the proteins presenilin 2 and 1, have been associated with senile plaque formation, and have also been found to explain a small number of familial AD cases (Gopinath, 1998; Mullan and Crawford, 1993; Selkoe, 1996).

A gene located on chromosome 19, which codes for ApoE (responsible for transport of lipids) has been shown to influence AD risk. The rare isoform ApoE 2 allele (7%) is associated with a reduced risk of AD development, the more common isoform ApoE 3 allele (78%) has not been associated with an effect on AD risk, but the ApoE 4 allele (15%) has been associated with an increased risk of AD development (Lendon

et al., 1997; Roses, 1996; Yankner and Mesulam, 1991). ApoE 4 has been reported to promote amyloidogenesis (Castaño *et al.*, 1995; Kindy *et al.*, 1995). It is reported that ApoE may be involved in transporting β-amyloid from the brain, but the ApoE 4 allele may result in less efficient removal of β-amyloid, thus contributing to senile plaque formation (Evans, 2001). This may explain the role of ApoE 4 in AD pathology, although other mechanisms may also occur. For example, the reduction in ChAT activity in the cortex of AD patients has been found to be proportional to the ApoE 4 allele, indicating ApoE 4 may be associated with cholinergic degeneration (Soininen *et al.*, 1995).

It has been estimated that genetic links may account for approximately 50% of AD cases (Plassman and Breitner, 1996). In the other AD cases, environmental factors may be more significant contributing factors, perhaps by interaction with undetermined genetic factors.

More recently, genes on chromosome 10 have also been associated with senile plaque formation, and increase the risk of developing late onset AD (Butcher, 2000; Majores *et al.*, 2000). In view of this discovery, it is apparent that genetic influences may be more significant in AD risk than has been suggested previously, and that the genetic association with senile plaque formation indicates that senile plaques are a significant factor in the early pathogenesis of AD.

1.1.2.2 Alterations in the Blood-Brain Barrier

The blood-brain barrier (BBB) is composed of specialised cerebral endothelium (capillary and arteriolar endothelium), which restricts the passage of molecules from blood to the brain (Bradbury, 1984; Conford, 1985). It has been proposed that injury, or abnormalities in the BBB may be involved in AD pathogenesis. Evidence suggests that in AD, the BBB becomes leaky due to gaps in tight junctions, and there are significant changes in glucose transport, events that may contribute to cerebral hypoperfusion and neuronal death (de-Figueiredo *et al.*, 1997). Serum proteins (e.g. amyloid P component) are reported to be detected in senile plaques (Perlmutter, 1994), which indicates BBB impairment may allow toxins to reach the brain. It has been proposed that it is β-amyloid that promotes the initial degeneration of the vasculature of the BBB (Kalaria, 1997; Perlmutter, 1994; Vinters and Pardridge, 1986; Wisniewski *et al.*, 1997). Therefore BBB dysfunction may not be a cause of

AD, but may participate in the pathological processes associated with AD progression.

1.1.2.3 Environmental Factors

Environmental factors that have been proposed to cause AD include infections (e.g. herpes simplex virus) and exposure to toxic agents (e.g. excessive alcohol and aluminium) (Gopinath, 1998; Haase *et al.*, 1986; Royston *et al.*, 1992; Savory *et al.*, 1996). High levels of calcium, silicon, iron and aluminium have been located in brains of AD patients, and have therefore been linked with AD development, perhaps due to increased oxidative stress. It is unknown if the presence of metal ions in plaques of AD patients contribute to their development or if they accumulate after plaques have formed.

Dietary aluminium intake has been proposed to be associated with an increased risk of AD (Rogers and Simon, 1999). Aluminium increases BBB permeability (Banks and Kastin, 1983), which could result in neurotoxins reaching the brain and causing neurodegeneration. It has been reported that aluminium can induce the formation of neurofibrillary tangles, similar to those identified in AD, *in vivo* (Barker and Branford, 1991). A rare form of dementia has also been identified in dialysis patients, which has been associated with excess aluminium in the dialysing fluid, and which shows similar pathology to AD (Barker and Branford, 1991; Candy *et al.*, 1992; Flaten *et al.*, 1996; Scholtz *et al.*, 1987). These occurrences suggest aluminium may contribute to AD development, but this theory has been questioned. For example, in cases of aluminium toxicity, AD pathology has not been established and, in workers in an aluminium reduction plant, no significant increase in AD risk was found (Barker and Branford, 1991; Moulin *et al.*, 2000).

Head injury is reported to trigger the formation of β-amyloid plaques, for example, dementia pugilistica has been linked to the repeated head trauma incurred by boxers (Roberts *et al.*, 1991; Royston *et al.*, 1992). The occurrence of β-amyloid plaque formation in dementia induced by both genetics and head injury, reinforces the significance of plaque formation in dementia pathogenesis.

Diet has also been linked to AD. Some studies have shown a correlation between low serum concentrations of folate and vitamin B_{12}, and high levels of homocysteine, which has been associated with an increased risk of AD (Clarke *et al.*, 1998; Lehmann

et al., 1999; Miller, 1999). Raised homocysteine levels are also associated with cardiovascular disease, which may also be a contributing factor in AD pathogenesis. Homocysteine has been found to potentiate β-amyloid-mediated toxicity in neuronal cultures (White *et al.*, 2001), suggesting that the hyperhomocysteinaemia associated with AD may contribute to the cytotoxic effects of β-amyloid. It is unknown if supplementing the diet with folate and vitamin B_{12} would protect against AD, and further investigation is necessary to establish this.

Copper deficiency has also been proposed to be a contributing factor to AD risk, and has been implicated in neuronal degeneration (Hartmann and Evenson, 1992). Further investigations are necessary to establish any dietary links with AD, but considering the multipathogenic nature of the disease, and the variety of proposed causes, diet is unlikely to be the only contributing factor in AD pathogenesis.

1.1.2.4 Other Proposed Contributing Factors

Alterations in cerebral microvasculature, cerebrovascular disease and hypertension have also been implicated as causes of dementia (Abe *et al.*, 1991; Buée *et al.*, 1994; Skoog, 1997). These occurrences may cause vascular insufficiency (e.g. as a result of cerebral infarcts) or BBB dysfunction, thus allowing toxins to permeate through to the brain. A specific role for these conditions in AD pathogenesis remains to be established.

It has been proposed that the neurotransmitter glutamic acid may cause neurodegeneration. Increased levels of glutamic acid may stimulate highly sensitive N-methyl-D-aspartate (NMDA) and amino-3-hydroxy-5-methylisoxasole-4-propionic acid (AMPA) receptors, resulting in glutamatergic neuronal death (Ossowska, 1993). It has not been established if glutamic acid plays a significant role in the initiation of AD, but it is likely to participate in the pathological processes in AD. For example, peptides identified in β-amyloid protein enhanced the neurotoxicity of glutamic acid through NMDA and kainite receptors (Mattson *et al.*, 1992).

Other factors that have been linked with AD development include oxidative stress to neurons, inflammatory processes within the CNS and alterations in hormone levels with the aging process. These factors are further discussed in relation to potential AD treatment (refer to 1.2.2, 1.2.3 and 1.2.4).

1.2 Therapeutic Strategies for Management of Alzheimer's Disease

It is apparent that AD involves multiple pathogenic events, including neurotransmitter disturbances, senile plaque and neurofibrillary tangle formation, and oxidative and inflammatory processes. Management of AD should address the known underlying pathologies in order to improve symptoms, and perhaps to prevent and delay disease progression.

1.2.1 Enhancement of Cholinergic Function

The presynaptic synthesis of ACh (1) from choline and acetyl coenzyme A in neurons is catalysed by the enzyme ChAT (Marshall and Parsons, 1987). Once synthesised, ACh (1) is stored in synaptic vesicles and is released upon neuronal stimulation into the synaptic cleft (Marshall and Parsons, 1987). ACh (1) may then stimulate post-synaptic receptors (or pre-synaptic receptors to regulate ACh (1) release) before being hydrolysed to yield choline and acetate, under the action of AChE. Improving cholinergic function by targeting the processes involved in cholinergic neurotransmission is a rational approach to palliative treatment of AD.

However, cholinergic therapies have been relatively unsuccessful and when effective, only give moderate symptomatic improvement. Consequently, cholinergic enhancers may require use with other agents proposed to be relevant in AD therapy, to maximise cognitive benefits.

1.2.1.1 Precursors of Acetylcholine

Adequate availability of choline has been proposed to permit adequate ACh (1) synthesis for neurotransmission. Precursors of ACh (1) (e.g. choline and lecithin) have been investigated for their effects on synthesis and release of ACh (1), with a view to increasing ACh (1) release and cholinergic activity. Few studies have reported any significant beneficial effects on cognitive function, and only modest if any, beneficial effects were observed (Kumar *et al.*, 1998b).

Therapy failure may be due to impaired uptake mechanisms of choline causing the reduction in ACh (1) synthesis, and not due to insufficient choline supply. This is apparent as it has been reported that more choline occurs in the cerebrospinal fluid

(CSF) of AD patients than in patients without AD, and that choline levels increased with disease progression (Giacobini, 1993; Kumar *et al.*, 1998b). Therapy with ACh (1) precursors was also limited by side-effects, including gastro-intestinal disturbances such as nausea, vomiting and diarrhoea.

ACh (1) precursors have been combined with AChE inhibitors, with the aim of enhancing cholinergic effects. Combinations of the AChE inhibitors tacrine (7) and physostigmine (5) with lecithin did not promote additional cognition enhancing effects in AD patients (Chattellier and Lacomblez, 1990; Peters and Levin, 1979; Wettstein, 1983). Therefore, precursors of ACh (1) do not appear to be important for AD treatment.

1.2.1.2 Stimulation of Cholinergic Receptors

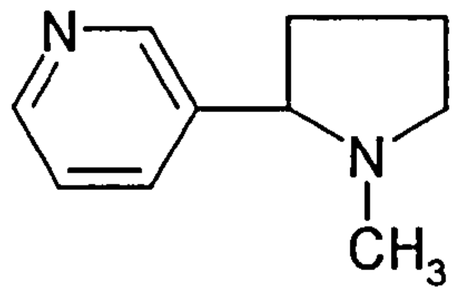
Cholinergic agonists are reported to facilitate learning and memory but cholinergic antagonists impair learning and memory (Blokland, 1996; Karczmar, 1995), thus cholinergic agonists may be appropriate in cognitive disorders such as AD.

Nicotinic Receptor Ligands

Behavioural studies have shown that nicotinic receptors, which are ligand-gated ion channels, participate in functions such as attention, memory and cognition (Mihailescu and Drucker-Collin, 2000). Nicotinic receptors have been shown to be reduced in cortical brain areas in AD (London *et al.*, 1989; Martin-Ruiz *et al.*, 2000; Nordberg, 1993; Perry *et al.*, 1990; Shimohama *et al.*, 1986); nicotine (2) is reported to upregulate nicotinic receptors and to increase ACh (1) release (Balfour and Fagerström, 1996; Flores *et al.*, 1992; Whitehouse and Kalaria, 1995). Thus nicotinic receptor agonists may enhance cholinergic neurotransmission in AD. Nicotine (2) treatment in various *in vivo* studies, including administration to rats with cholinergic brain lesions and in aged monkeys, has been shown to improve cognitive function (Buccafusco and Jackson, 1991; Decker *et al.*, 1992; Rezvani and Levin, 2001).

It is also reported that smoking may have protective effects against AD development and that administration of nicotine (2) to AD patients improved cognitive function (Graves and Mortimer, 1994; Newhouse and Kelton, 2000; Sahakian *et al.*, 1989; van Duijn and Hofman, 1991). However, some cohort studies have shown that smoking

shows either no association with AD risk, or moderately increases AD risk (Kukull, 2001; Merchant *et al.*, 1999; Tyas *et al.*, 2000). Perhaps a possible increased AD risk may be related to the consequences of smoking (e.g. free radical production and cerebrovascular disease). As several studies have also shown that nicotine (**2**) alone (in the absence of other chemicals associated with smoking) may enhance cognitive function, use of nicotine (**2**) or related compounds may be an approach in AD therapy.



Nicotine (**2**)

The cognition enhancing effects of nicotine (**2**) reported in animals and humans, may be due to nicotinic receptor stimulation. But nicotine (**2**) may also protect against AD by other mechanisms. Nicotine (**2**) has been shown to inhibit β-amyloid formation *in vitro* (Salomon *et al.*, 1996; Zamani and Allen, 2001), which may also explain the apparent protective effects of smoking against AD. However, one study that involved analysis of AD brains showed that smoking did not appear to protect against senile plaque formation (Ulrich *et al.*, 1997). Nicotine (**2**) has been reported to inhibit the neurotoxic effects of excitatory amino acids (e.g. glutamate) and to enhance the effects of nerve growth factor (NGF) (Akaike *et al.*, 1994; Whitehouse and Kalaria, 1995), which may explain the possible protective effects of smoking against AD and favourable effects on cognition.

Numerous nicotinic receptor subtypes have been identified (Williams *et al.*, 1994). For example, the α_7 and $\alpha_4\beta_2$ receptor subtypes have been found in brain regions important for cognition, including the cerebral cortex and hippocampus, and the compound GTS-21 is a relatively selective agonist for the α_7 receptor (and also interacts with the $\alpha_4\beta_2$ receptor) (Kem, 2000; Rezvani and Levin, 2001). GTS-21 is reported to enhance cognition *in vivo*, and was neuroprotective to neuronal cells exposed to β-amyloid *in vitro* (Kem, 2000). Thus, GTS-21 may be useful in AD therapy.

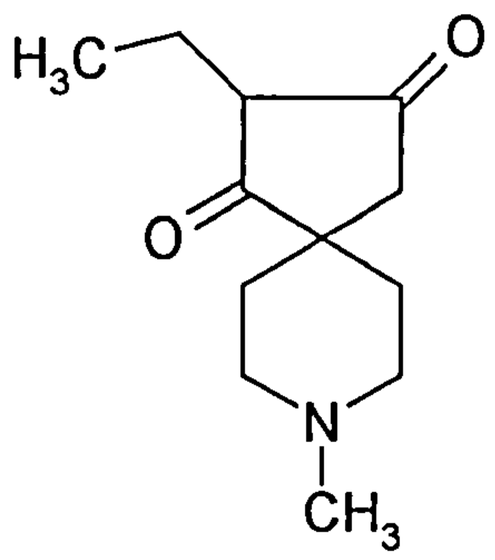
If nicotinic agonists continue to be thoroughly assessed, and consistently enhance cholinergic function or perhaps influence other mechanisms in AD patients, there is the opportunity for selective drug development to minimise adverse-effects associated

with nicotine (**2**) (e.g. cardiovascular effects (Benowitz, 1992)) and maximise efficacy. ABT-418, a nicotinic receptor agonist, has been shown to interact more selectively with the $\alpha_4\beta_2$ receptor subtype and was as effective as nicotine (**2**) in memory tasks *in vivo*, but with fewer adverse effects than nicotine (**2**) and, was neuroprotective *in vitro* (Arneric *et al.*, 1995). Therefore, selective nicotinic receptor agonists may have potential in future AD therapy.

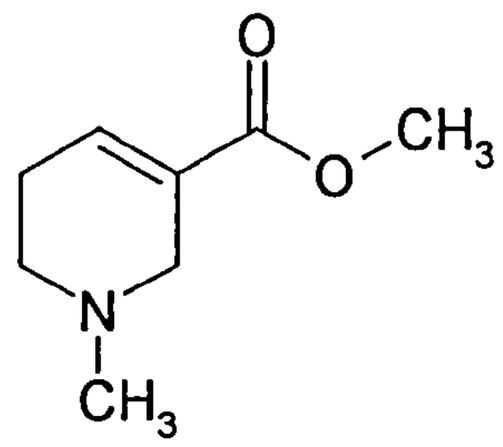
Muscarinic Receptor Ligands

Although muscarinic receptors, which are coupled to G-proteins, are not reported to be significantly reduced in AD (with the exception of the M_2 receptor subtype) (Nordberg, 1992; Price *et al.*, 1985), treatment with muscarinic agonists has not shown significant benefit in cognitive function.

Treatment with xanomeline, a muscarinic receptor agonist, showed some improvement in behavioural symptoms and cognition in AD patients in a double-blind, placebo-controlled trial, but 52% of patients discontinued treatment due to adverse effects, including gastro-intestinal side-effects (Bodick *et al.*, 1997). The muscarinic agonist RS-86 (**3**) also showed no significant benefits in cognitive function, which would suggest it is not appropriate in AD therapy (Bruno *et al.*, 1986; Hollander *et al.*, 1987; Mouradian *et al.*, 1988).



RS-86 (**3**)



Arecoline (**4**)

Arecoline (**4**), which has relative selectivity for M_1 receptors, only moderately improved cognitive function and recognition in AD patients (Raffaele *et al.*, 1996; Tariot *et al.*, 1988). Arecoline (**4**) is reported to poorly penetrate the BBB (Krogsgaard-Larsen *et al.*, 1989), which may explain the lack of significant benefits observed *in vivo*. The unpleasant side-effects reported to be associated with

muscarinic agonists (e.g. arecoline (**4**), pilocarpine, RS 86 (**3**)) may prevent their clinical use.

Muscarinic antagonists (e.g. the M_2 antagonists DX-116, AF-DX384 and AQ-RA 741) acting at presynaptic autoreceptors, at which ACh (**1**) regulates its own release, elevated ACh (**1**) levels *in vitro* and *in vivo* (Consolo *et al.*, 1987; Hoss *et al.*, 1990; Stillman *et al.*, 1993; Stillman *et al.*, 1996; Torocsik and Vizi, 1991; Wilson *et al.*, 1993). However, as it is the M_2 receptors which are reported be reduced in AD brains, M_2 receptor levels may not be sufficiently high to enable significant enhancement of cholinergic activity with these compounds.

It is reported that no muscarinc agonists with a high selectivity for a particular receptor subtype (M_1 - M_5) exist (Caulfield and Birdsall, 1998). Knowledge of the most relevant receptor subtype in AD treatment, and the development of highly selective muscarinic agonists (or presynaptic receptor antagonists) may be appropriate for AD management.

1.2.1.3 Inhibition of Acetylcholinesterase

The cholinesterases hydrolyse choline esters, with AChE being more specific for ACh (**1**) hydrolysis. The functions of butylcholinesterase (BuChE) are uncertain, including any possible involvement it may have in AD pathogenesis (Taylor, 1991). AChE, but not BuChE, has been shown to promote amyloid formation (Inestrosa *et al.*, 1996a; Inestrosa *et al.*, 1996b). AChE is bound to the pre- and post-synaptic membrane, where it hydrolyses ACh (**1**). Therefore the availability of ACh (**1**) released into the synaptic cleft may be prolonged, by inhibiting its hydrolysis by AChE through the use of AChE inhibitors.

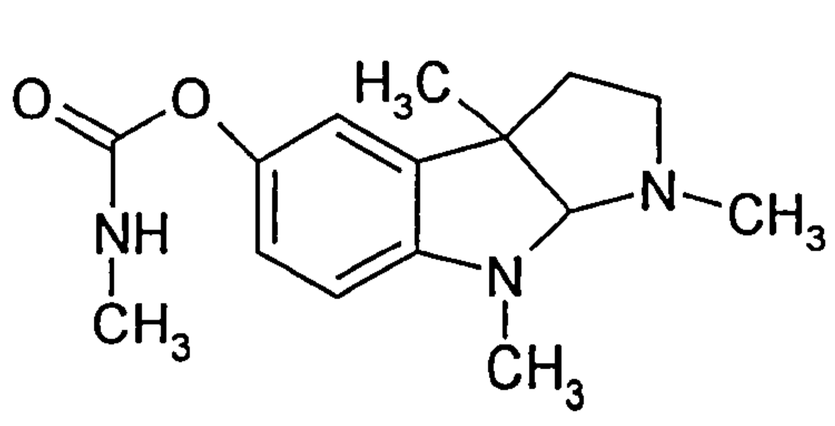
Six isoenzymes of AChE, which is a large glycoprotein, have been identified: globular monomers (G_1), dimers (G_2) and tetramers (G_4), and tailed asymmetric tetramers (A_4), double tetramers (A_8) and triple tetramers (A_{12}) (Brimijoin, 1983). The majority of AChE in nervous tissue is globular, with 80% - 90% in mammalian brain being in the G_4 form, and most of the remainder occurring as the G_1 form; tailed forms of AChE are reported to compose <1% of brain AChE (Brimijoin, 1983). In AD, a reduction in the tetrameric form (G_4) of AChE in the cortex and hippocampus is accompanied by a 300- 400-fold increase in asymmetric AChE, particularly A_{12}, and G_1 levels also increase (Arendt *et al.*, 1992; Fishman, *et al.*, 1986; Ogane *et al.*,

1992; Younkin *et al.*, 1986). The significance of the changes in the forms of AChE in AD is unknown.

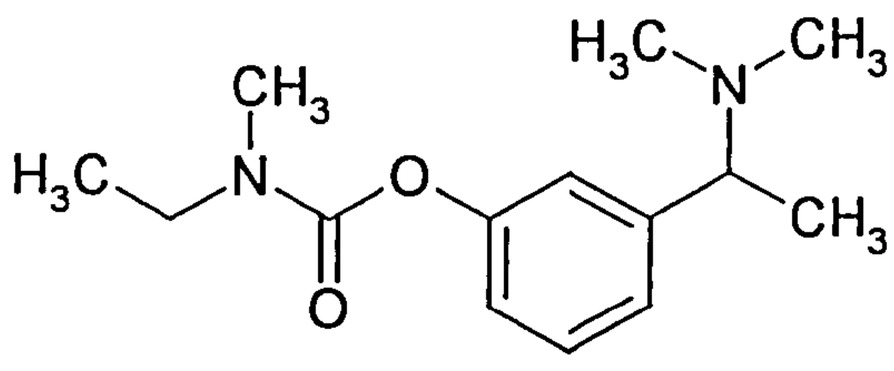
Physostigmine and Rivastigmine

Physostigmine (**5**) has been shown to inhibit both G_1 and G_4 AChE forms (Ogane *et al.*, 1992), the major AChE isoenzymes present in mammalian CNS. This effect indicates that physostigmine (**5**) inhibits CNS AChE but, as G_4 AChE levels are reported to be decreased in AD brains (Ogane *et al.*, 1992; Younkin *et al.*, 1986), there may be clinical implications regarding efficacy. An inhibitor that is more selective for the G_1 AChE form, may be more relevant in AD. Physostigmine (**5**) inhibited AChE and BuChE with similar potency (Otoguro *et al.*, 1997). Therefore, adverse effects associated with BuChE inhibition may also occur with physostigmine (**5**).

Physostigmine (**5**), a short-acting reversible AChE inhibitor, was reported to show cognitive benefits in both non-AD and AD patients (Davis and Mohs, 1982; McCaleb, 1990; Shu, 1998; Sitaram *et al.*, 1978), but clinical use may be limited by its short half-life, which would require multiple daily dosing. This is perhaps impractical in AD patients.



Physostigmine (**5**)



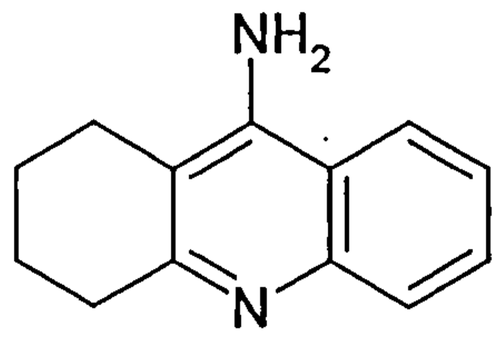
Rivastigmine (**6**)

Rivastigmine (**6**), an analogue of the alkaloid physostigmine (**5**), was licensed for use in the UK in 2000 for the symptomatic treatment of mild to moderately severe AD. Rivastigmine (**6**) improves cognition in AD patients, an effect that has been correlated with the level of AChE inhibition (Agid *et al.*, 1998; Cutler *et al.*, 1999; Grossberg and Desai, 2001; Kumar *et al.*, 1999; Spencer and Noble, 1998). Rivastigmine (**6**) preferentially inhibits the G_1 form of AChE (Polinsky, 1998), which may occur at elevated levels in AD. Greater selectivity for the G_1 form of AChE may enhance efficacy and minimise adverse effects. Inhibition of other forms of AChE in the

peripheral nervous system (e.g. in muscles and cardiovascular system) may increase the incidence of adverse effects, such as cramps and bradycardia. Rivastigmine (**6**) is reported to inhibit AChE in the cortex and hippocampus (Polinsky, 1998), brain areas involved in cognition. Rivastigmine (**6**) is therefore a more appropriate candidate for use in AD patients than physostigmine (**5**).

Tacrine

The first AChE inhibitor to be licensed for management of AD was tacrine (7), a centrally active reversible inhibitor, which requires dosing three times daily due to the relatively short half-life. Tacrine (7) is longer acting than physostigmine (**5**), therefore has some dosing advantages; tacrine (7) readily crosses the BBB and moderate improvement in symptoms was observed following its use in AD patients (Farlow *et al.*, 1993; Knapp *et al.*, 1994; Olin and Schneier, 1995; Summers *et al.*, 1986). Tacrine (7) may also enhance cholinergic neurotransmission by stimulating ACh (**1**) synthesis via an unknown mechanism (Doležal and Tuček, 1991).

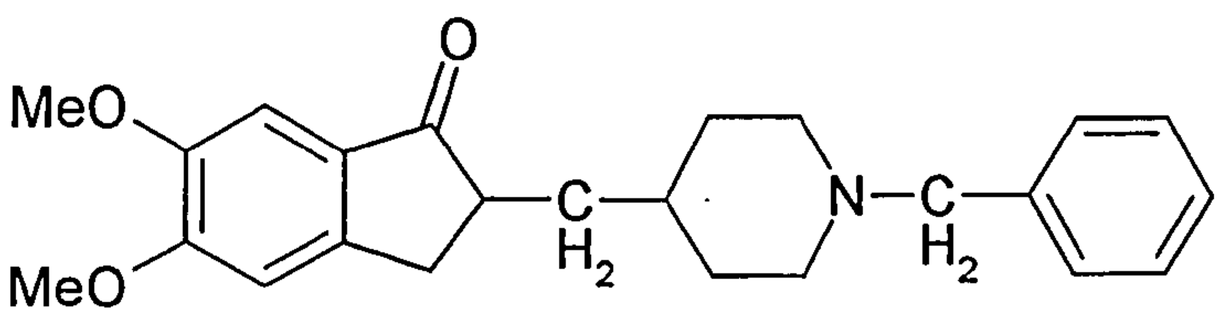


Tacrine (7)

Routine use of tacrine (7) is restricted by hepatotoxic side-effects (Hammel *et al.*, 1990; Watkins *et al.*, 1994). Both tacrine (7) and physostigmine (**5**) have been shown to antagonise scopolamine-induced impairment of cognitive function in rats (Yoshida and Suzuki, 1993), which is further evidence for their favourable effects on cholinergic function *in vivo*. Tacrine (7) and physostigmine (**5**) both have low bioavailability (17% - 37% and 3% - 8% respectively), compared to newer AChE inhibitors such as donepezil (**8**), rivastigmine (**6**) and galantamine (**9**) (40% - 100% bioavailability) (Nordberg and Svensson, 1998).

Donepezil

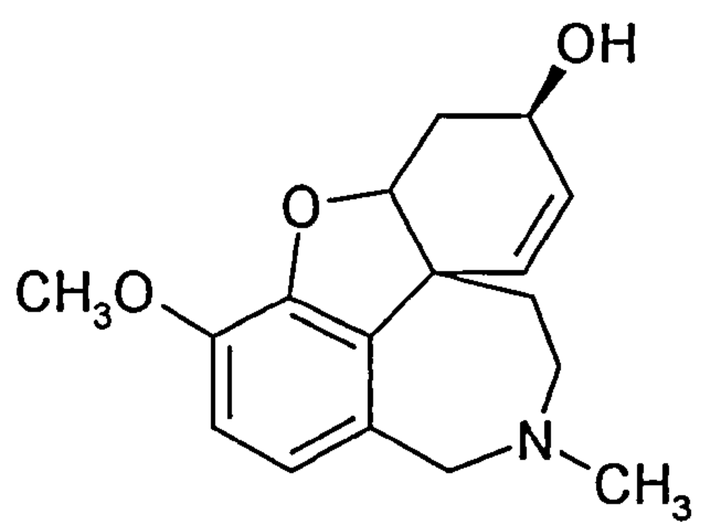
Donepezil (**8**), an inhibitor relatively selective for CNS AChE, was licensed for use in the UK in 1997 for the symptomatic treatment of mild to moderately severe AD. Severe hepatotoxicity is not associated with donepezil (**8**), and dosing is required once daily due to the drug's longer half-life (70hr - 80hr), which are advantages over tacrine (7). Donepezil (**8**) has also shown significant improvements in the cognitive functioning of AD patients (Barner and Gray, 1998; Melzer, 1998).



Donepezil (**8**)

Galantamine

Galantamine (**9**) is reported to be more selective for AChE than BuChE, and provides complete oral bioavailability (Bickel *et al.*, 1991; Fulton and Benfield, 1996; Harvey, 1995). Galantamine (**9**) is licensed in Europe for AD treatment and was well tolerated and significantly improved cognitive function when administered to AD patients, in a multi-centre randomised controlled trial (Wilcock *et al.*, 2000).



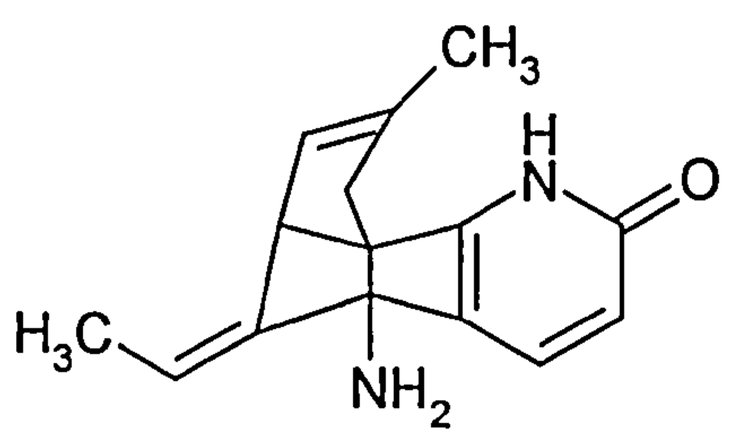
Galantamine (**9**)

Galantamine (**9**) is also reported to stimulate nicotinic receptors (Woodruff-Pak *et al.*, 2001), which may also enhance cholinergic function and memory. This additional activity, suggests galantamine (**9**) may have therapeutic advantages over other AChE inhibitors.

Other AChE Inhibitors for Potential AD Therapy

Several other AChE inhibitors have been investigated for their potential in AD therapy. E2020 inhibited brain AChE in rats following subcutaneous administration, and consequently increased ACh (**1**) levels in the cortex, but increases in levels of dopamine and noradrenaline were also observed (Giacobini *et al.*, 1996). These results indicate that dopaminergic and noradrenergic neurons may have been influenced by the E2020 stimulated cholinergic system, such interactions have previously been reported (Decker and McGaugh, 1991; Moroni *et al.*, 1983; Vizi, 1980), or that perhaps E2020 may independently interact with these neuronal systems to influence neurotransmitter levels.

The alkaloid huperzine A (**10**), isolated from the moss *Huperzia serrata*, which has been used in China for centuries to treat fever and inflammation, is also a reversible inhibitor of AChE (Ashani *et al.*, 1992; Laganière *et al.*, 1991; McKinney *et al.*, 1991; Wang *et al.*, 1986b). Huperzine A (**10**) is used in China for the symptomatic treatment of dementia, and is being investigated for international use (Houghton, 1999; Skolnick, 1997).

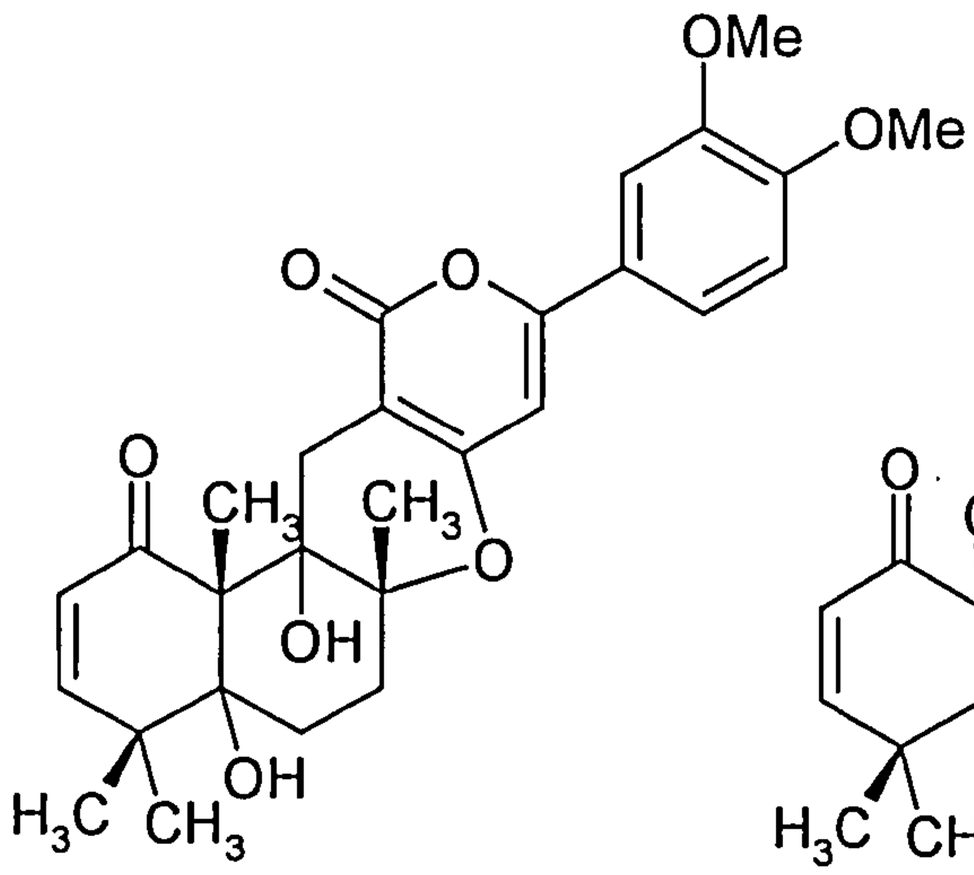


Huperzine A (**10**)

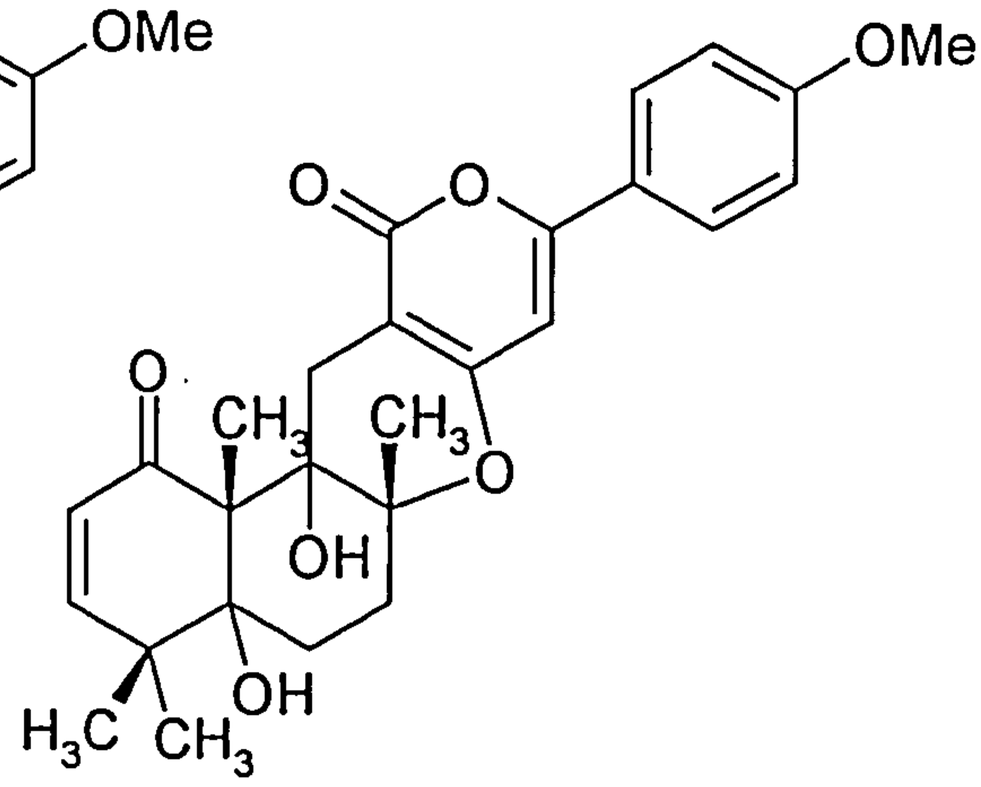
In a multi-centre, double-blind trial, huperzine A (**10**) significantly improved memory and behaviour in AD patients, and was reported to be more selective for AChE than BuChE and less toxic than donepezil (**8**) and tacrine (**7**) (Small *et al.*, 1997; Shu, 1998).

The AChE inhibitors in clinical use are natural alkaloids or structurally related to natural alkaloids, and contain nitrogen within a ring system. Arisugacins A (**11**) and B (**12**) are non-alkaloidal selective inhibitors of AChE (and were not selective for BuChE) from *Penicillium* spp. and have been shown to be 200-fold more potent than tacrine (**7**) at inhibiting AChE activity *in vitro* (Ōmura *et al.*, 1995; Otoguro *et al.*,

1997). The arisugacins may have potential in AD therapy, but further studies *in vivo* are necessary to establish efficacy and adverse-effects.

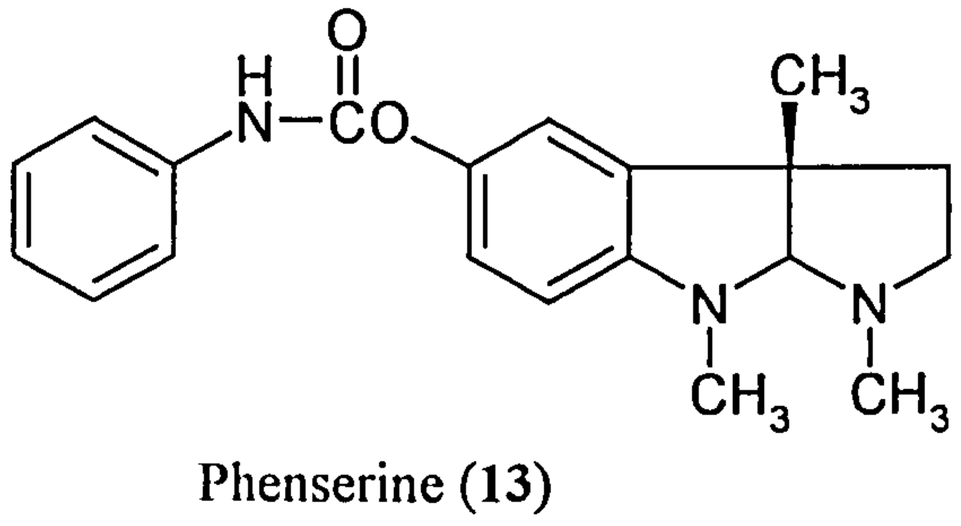


Arisugacin A (**11**)



Arisugacin B (**12**)

Phenserine (**13**), a derivative of physostigmine (**5**), is a selective inhibitor of AChE (>50-fold inhibitory activity for AChE than BuChE), with 100% bioavailability (Greig *et al.*, 2000). Phenserine (**13**) is also reported to reduce APP levels by interfering in transcription processes, and subsequent senile plaque formation (Evans, 2001).



Phenserine (**13**)

Administration of phenserine (**13**) to rats with learning impairment (induced by a NMDA receptor antagonist) resulted in improved cognitive function (Patel *et al.*, 1998). This indicates that phenserine (**13**) may additionally influence glutaminergic function, and is a potential candidate for AD therapy.

Assessment of the Use of AChE Inhibitors in Alzheimer's Disease

The efficacy of AChE inhibitors is limited by several factors. Functional cholinergic neurons are necessary for the effectiveness of drugs that aim to improve cholinergic neurotransmission, which may limit efficacy, particularly in the later stages of AD when neuronal degeneration is significant. The low levels of AChE reported to occur in AD (Arendt, *et al.*, 1992; Atack *et al.*, 1983; Hammond and Brimijoin, 1988) may also limit successful treatment.

The use of AChE inhibitors for AD treatment has been proposed to actually induce harm rather than favourable effects; AChE levels in AD are imbalanced, which may reflect an underlying pathology of AD in addition to the adverse effects reported (Shen, 1996). Inhibition of AChE activity may further disrupt AChE activity, causing further imbalance and perhaps promote disease progression. The adverse effects induced by AChE inhibitors may cause harm. For example, anticholinesterase (antiChE) organophosphates are reported to induce myopathy, myocardial pathology and polyneuropathy (Millard and Broomfield, 1995), but these effects may not be associated with the AChE inhibitors designed for AD therapy. However, other potentially serious adverse effects have been associated with AChE inhibitors (e.g. donepezil (8)), including bradycardia (perhaps mediated by peripheral M_2 receptors (Lazartigues *et al.*, 1998)) and seizures; in addition hallucinations, aggressive behaviour and agitation have been reported to occur (CSM and MCA report, 1999).

In conclusion, AChE inhibitors appear to offer some short-term benefits in cognition in some, but not all, AD patients, and clinical use may be limited by adverse effects (e.g. nausea, vomiting and diarrhoea). Thus, antiChE therapy in AD should continue to be evaluated to justify routine use, but AChE inhibitor use in AD may be more appropriate with the development of more selective AChE inhibitors.

1.2.2 Oestrogen Replacement Therapy

The oestrogen (or estrogen) receptor (ER) is a member of the steroid receptor superfamily of transcription factors, which also includes receptors for vitamin D, thyroid hormone and retinoic acid. The ER has been found to occur as two subtypes: ERα and ERβ. The ER is responsible for regulation of gene expression, for genes

involved in tissue growth and differentiation. ERα and ERβ have different transcriptional activation properties.

ERα is predominantly expressed in breast, uterus, pituitary and ovary but ERβ is predominantly expressed in prostate, bone, vascular tissue and uterus in rats (Dechering *et al.*, 2000; Kuiper *et al.*, 1997; Mason, 2001). ERα and predominantly ERβ have been detected in the hippocampus, an area important for cognition in the monkey, and oestrogen treatment was suggested to modulate behaviours and functions mediated by the amygdala (Osterlund *et al.*, 1998; Register *et al.*, 1998). More recently, ERα has also been detected in rat cerebral cortex and human amygdala, hypothalamus, cerebral cortex and hippocampus (Butler *et al.*, 1999; Osterlund *et al.*, 2000), which suggests that ERα modulation may not only be related to endocrine function, but also to cognitive function.

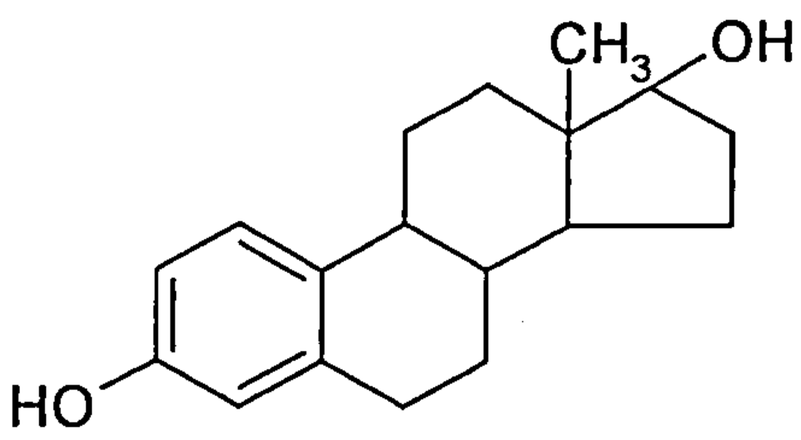
AD is reported to affect 1.2 - 3.5 times more women than men, an effect that has not been associated with greater longevity in women (Birge, 1997; McGuffey, 1997). Although oestrogen levels are lower in men than women, levels are reported to be maintained at a constant level throughout life in men due to intracerebral metabolism of testosterone to oestrogen, until testosterone levels decline. Epidemiologic studies show that AD incidence increases in women after the age of 65 years, but gender differences are not associated with early onset AD, in which oestrogen levels are generally at constant levels in both men and women (Birge, 1996; Paganina-Hill and Henderson, 1996; Payami *et al.*, 1996).

Several studies indicate that oestrogen (or estrogen) replacement therapy (ERT) may have favourable effects in the prevention and treatment of AD. ERT in women is associated with a reduced risk of developing AD and, oestrogen treatment in women with AD is reported to enhance cognitive function and also enhance the response to tacrine (7) (Birge, 1997; Fillit *et al.*, 1986; Honjo *et al.*, 1989; Ohkura *et al.*, 1994; Schneider *et al.*, 1996). ERT is also reported to enhance cognitive function in women without AD. For example, high serum oestrogen concentrations in women have been associated with less impairment of cognitive function than low oestrogen levels, and ERT improved memory skills in post-menopausal women (Kampen and Sherwin, 1994; Yaffe *et al.*, 2000). ERT also improved cognitive function in ovariectomised rats (Birge, 1997). However, some studies did not show cognitive improvement in AD patients (Mulnard *et al.*, 2000).

The mechanisms by which oestrogens may protect against AD are unclear, but may be mediated by ERs in the basal forebrain and hippocampus, effects on neurotransmitter systems, modulation of neurotrophic growth factor levels, maintenance of synaptic density, enhancement of cerebral blood flow, anti-oxidant effects or other unknown mechanisms.

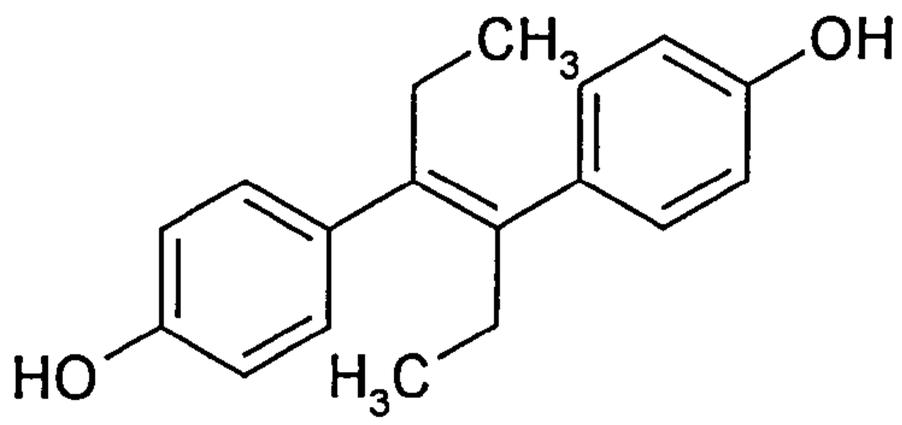
1.2.2.1 Anti-Oxidant Activity of Oestrogens

17β-Oestradiol (E2) (**14**) protected hippocampal neuronal cells from oxidative stress-induced cytotoxicity following exposure to β-amyloid, H_2O_2 or glutamate *in vitro*; other hormones including progesterone, aldosterone and corticosterone, were not neuroprotective (Behl *et al.*, 1995). Other studies have also shown that oestrogens (e.g. estriol and E2 (**14**)) are anti-oxidant and that other steroids (e.g. progesterone and testosterone) were not anti-oxidant (Mooradian, 1993). E2 (**14**) and 2-hydroxy-E2 have also been shown to be good inhibitors of lipid peroxidation (Ruiz-Larrea *et al.*, 1994; Wiseman *et al.*, 1990). These results suggest that oestrogens may protect against the oxidative processes, including peroxidative damage to cell membranes, associated with AD (refer to 1.2.4).



17β-Oestradiol (**14**)

Several synthetic oestrogens (e.g. diethylstilboestrol (**15**)) and some anti-oestrogens (e.g. ICI 164, 384 and tamoxifen) are also reported to be anti-oxidant and were inhibitors of lipid peroxidation *in vitro* (Wiseman *et al.*, 1990; Wiseman and Halliwell, 1993; Wiseman, 1994).



Diethylstilboestrol (**15**)

1.2.2.2 Effect of Oestrogens on Neurotransmitter Systems

Steroids have been shown to affect various neurotransmitter systems in the CNS, which may also contribute to the proposed beneficial effects of oestrogens in AD. Steroids are reported to act non-transcriptionally in neurons and may influence neurotransmission and synaptic function (McEwan, 1991; Moss *et al.*, 1997), which suggests they may have functions other than cell growth and differentiation.

E2 (**14**) treatment in ovariectomised rodents increased dopamine turnover and striatal dopamine release *in vivo* (Becker, 1990; Di Paolo *et al.*, 1985). It is not yet understood how E2 (**14**) may influence dopamine release, but it may involve binding to an external ER binding site in the striatum (Xiao and Becker, 1998). E2 (**14**) also increased striatal dopamine receptors in rat brain *in vivo* (Hruska, 1986). The potential for oestrogenic compounds to enhance dopamine release and upregulate dopamine receptors may be relevant in AD and also Parkinson's disease.

Other neurotransmitter systems which have been influenced by E2 (**14**) include GABAergic, glutamatergic, noradrenergic and serotonergic systems; both increases and decreases in receptor densities have been observed (Biegon *et al.*, 1983; François-Bellan *et al.*, 1989; Herbison *et al.*, 2000; McEwen *et al.*, 1995; Moss *et al.*, 1997; Woodley *et al.*, 2000). Further studies are required to confirm these effects and identify any relevance to AD. E2 (**14**) also decreased monoamine oxidase (MAO)-A activity in rat brain (Luine and McEwen, 1977). This suggests that E2 (**14**) may suppress MAO-A activity, thus reducing degradation of 5-HT, which may be relevant in AD and depression.

Perhaps more relevant to AD, cholinergic neurons in the basal forebrain are reported to have ERs that are co-localised with NGF receptors (Sohrabji *et al.*, 1994; Toran-Allerand *et al.*, 1992). This indicates that oestrogens may influence cholinergic function and perhaps regulate NGF. E2 (**14**) has been found to increase ChAT

activity in various brain regions, including the basal forebrain and hippocampus in rats (Kaufman *et al.*, 1988; Luine, 1985), so may enhance cholinergic function. The basal forebrain of male rats did not show increased ChAT activity in response to E2 (**14**) treatment, which may reflect the differences in basal forebrains between male and female rats (females have smaller and more densely packed cholinergic neurons that males) (McEwen *et al.*, 1995), or perhaps differences in ER distribution.

1.2.2.3 Oestrogens and β-Amyloid

In vitro, E2 (**14**) promotes the metabolism of APP to β-amyloid peptides that are less likely to form senile plaque deposits (Jaffe *et al.*, 1994). E2 (**14**) has also been proposed to modulate inflammatory responses involved in senile plaque formation (Birge, 1997). β-Amyloid induced cytotoxicity *in vitro* was prevented by E2 (**14**), via interaction with ERα (Kim *et al.*, 2001). ERα ligands may therefore be appropriate for reducing β-amyloid-induced cytotoxicity in AD, but it cannot be excluded that other favourable effects associated with ERT may be mediated by ERβ, or perhaps receptor independent mechanisms. E2 (**14**) also enhanced the uptake of β-amyloid by microglia *in vitro*, which may result in enhanced clearance of β-amyloid in AD, thus reducing senile plaque formation (Li *et al.*, 2000).

The environmental oestrogen, bisphenol A, also protects neurons from β-amyloid- and glutamate-induced cytotoxicity *in vitro* (Gursoy *et al.*, 2001). Therefore synthetic oestrogens may also have potential in cognitive disorders.

Further research regarding the use of ERT in AD patients is necessary to establish the potential benefits. However, there is significant evidence to suggest that ERT may have a preventative effect against AD development, and may be useful in slowing disease progression. Consideration must also be given to the potential adverse effects of ERT (e.g. carcinogenesis). Phyto-oestrogens (e.g. soy isoflavones) are also reported to offer protection against AD development (Pharm. J., 2001) and may act similarly to ERT or via different mechanisms. Phyto-oestrogens have not been associated with the carcinogenic effects of ERT, and may be a more appropriate alternative in the prevention of AD.

1.2.3 Anti-Inflammatory Drugs

The inflammatory processes that occur in AD are complex, and the pathology remains poorly understood. Pro-inflammatory cytokines (e.g. tumour necrosis factor (TNF) and interleukins (IL-1 and IL-6)), complement proteins (e.g. C1q) and acute phase proteins (e.g. α-1-antitrypsin) have been identified in senile plaques (Dickson and Rogers, 1992; McGeer and McGeer, 1995; Schenk *et al.*, 1995). AD pathogenesis is reported to include activation of the classical complement system, expression of complement and cytokine receptors by microglia, and upregulation of toxic cytokines (Eikelenboom *et al.*, 1989; McGeer and McGeer, 1997; McGeer and McGeer, 1999a; McGeer and McGeer, 1999b; Rogers *et al.*, 1996; Vandenabeele and Fiers, 1991).
The co-localisation of inflammatory mediators with AD lesions (e.g. senile plaques) indicates that inflammatory processes may mediate neuronal damage, or may be a consequence of lesion formation, or both.

1.2.3.1 Acute Phase Reactants

The occurrence of acute phase reactants in AD brain suggests that local inflammatory processes may be involved in AD pathogenesis. The acute phase reactants associated with inflammation in AD include α-1-antichymotrypsin (ACT), α-2-macroglobulin, α-1-antitrypsin, serum amyloid P (SAP) and C-reactive protein (Rogers *et al.*, 1996); ACT and α-2-macroglobulin have been identified in senile plaques (Rozemuller *et al.*, 1990). ACT and SAP have been reported to alter the rate of aggregation of β-amyloid into the neurotoxic form, and ACT promoted β-amyloid deposition *in vivo* (Nilsson *et al.*, 2001; Rogers *et al.*, 1996). SAP is a significant component of senile plaques and has been associated with neurofibrillary tangles (Akiyama *et al.*, 1991; Pepys *et al.*, 1982).

The association of these mediators with senile plaque formation, and perhaps neurofibrillary tangle formation, suggests that they are contributors to disease progression.

1.2.3.2 Complement Proteins

The complement system is designed to promote phagocytosis and destroy foreign matter. The classical complement system is reported to be activated in AD brains (McGeer and McGeer, 1999b). Complement proteins associated with senile plaques and neurofibrillary tangles in AD include C1 - C9 (McGeer and McGeer, 1999b; Rogers *et al.*, 1996).

β-Amyloid may initiate the complement pathway, which may explain the inflammatory processes that occur in AD brain. β-Amyloid may bind to C1q, which may then activate the complement system (Webster *et al.*, 1997a). The activation of the complement pathway by β-amyloid was potentiated *in vitro* by ApoE 4, but not ApoE 2 or ApoE 3 (McGeer *et al.*, 1997). Individuals with the ApoE 4 allele, a risk factor for AD, may therefore experience significant inflammatory events in the brain, which may contribute to further neuronal degeneration. C1q itself also accelerates the aggregation of β-amyloid (Webster *et al.*, 1994). Therefore, inhibition of Clq binding to β-amyloid may be a target for reducing the inflammatory processes in AD, to minimise neuronal damage.

C1 activation cleaves C2 and C4 to generate a series of other complement proteins, and eventually C5b may combine with C6, C7, C8 and C9 to form C5b-9, the membrane attack complex (MAC), which inserts into membranes and causes lysis and death (McGeer and McGeer, 1999b). MAC has been reported to occur in degenerated neurons containing neurofibrillary tangles in the AD brain (Schenk *et al.*, 1995; Webster *et al.*, 1997b).

Synthesis of complement proteins occurs in the pyramidal neurons in cortical brain regions (Shen *et al.*, 1997). Rat pyramidal neurons express mRNA for cyclo-oxygenase-2 (COX-2), involved in eicosanoid generation (Tocco *et al.*, 1997), which may also explain the involvement of COX in inflammatory processes in AD.

Therefore, regulation of complement protein activity could be one approach to manage inflammation in AD, but this has not proved successful (Yasojima *et al.*, 1999a).

1.2.3.3 Cytokines

Inflammatory cytokines amplify and sustain inflammation and immune responses, and may be produced in the CNS by cells, including astrocytes, microglia and neurons (McGeer and McGeer, 1997). Activated T-cells may also produce cytokines and cross the BBB, and have been located in lesioned areas of AD brain (McGeer and McGeer, 1995). The cytokines identified as markers of inflammation in AD include IL-1, IL-6 and TNF.

The cytokine IL-1 is present in non-diseased brain where it is involved in the acute phase response and immune function. The APP gene is reported to be induced by IL-1 *in vitro*, and IL-1 immunoreactivity is increased in AD (Griffin *et al.*, 1989; Royston *et al.*, 1992), indicating that IL-1 over-expression in AD may increase APP synthesis and subsequent plaque deposition. IL-1 and TNF have been reported to regulate NGF synthesis, and increased NGF-mRNA *in vitro* (Lindholm *et al.*, 1987). The sprouting of neuronal axons and dendrites, associated with Alzheimer's pathology, may be explained by the neurotrophic effects of IL-1 (or TNF) stimulated NGF. It has also been proposed that IL-1 and TNF may induce IL-6, which in turn may induce α_1-antichymotrypsin, which is also a component of senile plaques (Vandenabeele and Fiers, 1991). IL-1 induced COX-2 expression and prostaglandin (PG) E_2 secretion in human neuroblastoma cells *in vitro* (Hoozemans *et al.*, 2001a). This suggests that IL-1 may also upregulate COX-2 expression in AD, and generate damaging inflammatory mediators.

IL-1 receptor antagonists may therefore be of therapeutic value in AD, and may also inhibit neuronal damage induced by cerebral ischaemia; in rats IL-1 expression was induced by brain ischaemia (Minami *et al.*, 1992). It has also been reported that intracerebroventricular administration of an IL-1 receptor antagonist to rats significantly reduced APP density (Royston *et al.*, 1992).

1.2.3.4 Immunoglobulins

Intercellular adhesion molecule-1 (ICAM-1) accumulates in microvascular endothelial cells and in senile plaques, which indicates that the infiltration of lymphocytes into the brain may occur in AD (Akiyama *et al.*, 1993; Verbeek *et al.*, 1994). However, ICAM-1 is also produced in astrocytes (Akiyama *et al.*, 1993;

Rosenman *et al.*, 1995), suggesting that the inflammatory processes in AD may also originate in the CNS.

1.2.3.5 Microglia and Astrocytes

Postmortem analysis of the brains of AD patients show evidence of inflammatory processes, including the presence of reactive microglia, which are cells involved in inflammatory responses in the CNS, compared to control tissue; microglia in AD are co-localised with senile plaques (Dickson and Rogers, 1992; DiPatre and Gelman, 1997; Lue *et al.*, 1996).

Microglia express numerous receptors, including complement receptors, immunoglobulin receptors, ICAM-1 receptors and antigens, and may be activated by various mediators, including complement proteins, cytokines and also β-amyloid (McGeer and McGeer, 1995; McGeer and McGeer, 1999b). Activated microglia may produce nitric oxide (NO), superoxides, glutamate, prostaglandins and possibly other inflammatory mediators and neurotoxins (McGeer and McGeer, 1999b), which may also contribute to neuronal death. In transgenic mice, both microglia and astrocytes increased in the presence of β-amyloid, and were closely associated with β-amyloid deposits (Matsuoka *et al.*, 2001).

Astrocytes, which are involved in the maintenance of synaptic function, are believed to be a source of arachidonic acid (AA) derivatives and other inflammatory mediators in the brain. Astrocyte proliferation may be stimulated by the elevated IL-1 levels in AD, resulting in secretion of various growth factors, interleukins, prostaglandins, leukotrienes, thromboxanes, ACT and complement factors (McGeer and McGeer, 1995; Walker *et al.*, 1998).

Prostaglandins (synthesised via COX) may potentiate glutamate toxicity by inhibiting re-uptake of glutamate by astrocytes (Breitner *et al.*, 1996b). Inhibitors of enzymes in the AA cascade (e.g. COX) may therefore be of therapeutic value in AD. Senile plaque formation in AD brain is reported to disturb the functions of astrocytes (Kerokoski *et al.*, 2001). This may result in impaired synaptic function and perhaps altered secretion of toxic mediators from astrocytes. Astrocytes have also been located in mature plaques (Frederickson, 1992), which is perhaps associated with their production of ApoE (Mrak *et al.*, 1995).

1.2.3.6 Origins of Inflammatory Processes

There is considerable evidence that inflammatory mechanisms occur in AD, but the reasons why these inflammatory processes occur are unclear. Immunological processes may explain the inflammatory mechanisms; perhaps impaired BBB integrity may allow immunological reactants to reach the brain. BBB permeability is reported to be impaired in neuroinflammatory diseases including AD, and it has been hypothesised that this may permit inflammatory mediators and other neurotoxins to enter the brain and perhaps initiate AD pathogenesis (De Vries *et al.*, 1997). It is possible however, that BBB impairment occurs during AD progression, as a result of inflammatory mechanisms.

Brain injury, arising from ischaemia, head injury or senile plaque formation may also contribute to inflammatory processes. For example, IL-1 expression is induced following brain injury or cerebral ischaemia (Minami *et al.*, 1992). An immune response to toxins, including senile plaque components and neurofibrillary tangles, may stimulate the inflammatory processes in the AD brain. Further neuronal death and senile plaque formation may then exacerbate inflammation resulting in continuous disease progression.

β-Amyloid stimulates cytokine production, which in turn stimulates APP production (Araujo and Cotman, 1992; Forloni *et al.*, 1992; Gitter *et al.*, 1993), which may promote further β-amyloid plaque formation, in a cyclic mechanism. β-Amyloid has also been reported to induce microglial phagocytosis, expression of inducible NO synthase and NO production, and neuronal loss in rat CNS *in vivo* (Weldon *et al.*, 1998).

It has also been proposed that viral infections may initiate an inflammatory response in the CNS, leading to senile plaque formation (Dickson and Rogers, 1992; Vandenabeele and Fiers, 1991), but this remains to be established in relation to AD.

1.2.3.7 NSAIDs

It is evident that inflammation is not the only pathogenic mechanism that occurs in AD. However, it is apparent that inflammatory processes may contribute to neurodegeneration and perhaps accelerate disease progression, therefore the use of anti-inflammatory agents may be important as part of AD therapy.

There are several studies that show that non-steroidal anti-inflammatory drugs (NSAIDs) may reduce the risk of developing AD, and that patients with rheumatoid arthritis, who often use NSAIDs, have a lower incidence of AD (Breitner, 1996a; Breitner *et al.*, 1995; Jenkinson *et al.*, 1989; McGeer *et al.*, 1990; McGeer *et al.*, 1996). The relative risk of AD decreases with increased duration of NSAID use (Stewart *et al.*, 1997).

It could be proposed that individuals with a genetic predisposition to rheumatoid arthritis may also have a genetically associated lower risk of developing AD. This could explain the apparent inverse relationship between AD and rheumatoid arthritis. However, use of dapsone, which is an anti-leprosy drug with anti-inflammatory activity, has also been associated with a lower incidence of AD (McGeer *et al.*, 1992). This suggests that anti-inflammatory therapy may be successful in delaying AD progression. This is also suggested by the results of a study in which treatment with indomethacin in a six month, double-blind, placebo-controlled trial, showed cognitive improvement in AD patients (Rogers *et al.*, 1993).

The pharmacological action of NSAIDs in the brain that may protect against AD remains unclear. COX inhibitors suppress prostaglandin synthesis (prostaglandins inhibit astrocyte reuptake of the neurotoxin glutamate), which may suppress the potentiation of glutaminergic transmission and so protect against excitotoxic cell death (Breitner, 1996b); over-stimulation of neuronal glutamate receptors, arising from excessive glutamate release, is neurotoxic (Rothman and Olney, 1986). It has also been shown that NSAIDs are neuroprotective *in vitro* (Klegeris *et al.*, 1999), which may explain their beneficial effects against AD. NSAIDs are also reported to prevent APP over-expression and over-production of amyloidogenic peptides (Lee and Wurtman, 2000), which indicates they may inhibit senile plaque formation.

Although the potential protective effects of NSAIDs against AD progression are well documented (Breitner, 1996a; Breitner, 1996b; Breitner *et al.*, 1995; Klegeris *et al.*, 1999 McGeer *et al.*, 1996; Rogers *et al.*, 1993), NSAID use, particularly in long-term therapy, is restricted by adverse effects (e.g. gastro-intestinal bleeding and ulceration) and potential interactions with other drugs. Consequently, there is a need for effective anti-inflammatory agents, which have advantages over the NSAIDs in terms of the adverse effect profile and efficacy.

NSAIDs are inhibitors of COX-1 and COX-2, but COX-2 inhibition may be more relevant to AD treatment. COX-2 may contribute to neuronal vulnerability, and is

reported to be expressed in the neocortex, which is involved in cognitive function (Felician *et al.*, 1997; O'Banion, 1999). COX-2 was upregulated in astrocytes associated with β-amyloid deposits in transgenic mice, and also stimulated β-amyloid production *in vitro* (Kadoyama *et al.*, 2001; Matsuoka *et al.*, 2001). Thus, COX-2 may participate in senile plaque formation AD. COX-2 levels were substantially upregulated in lesioned areas of AD brain, but COX-1 levels were only slightly upregulated (Yasojima *et al.*, 1999b). Therefore COX-2 inhibitors may be more appropriate for use in AD, particularly as the adverse effects of NSAIDs have been associated with COX-1 inhibition. But, COX-1-positive microglia associated with senile plaques have been identified in AD brains (Hoozemans *et al.*, 2001a). This suggests that COX-1 inhibition may also be relevant in AD therapy. Further investigations regarding the potential cognitive benefits of COX-1 and COX-2 inhibitors in AD are therefore warranted.

Prednisone treatment in patients with AD did not provide cognitive benefits, but showed behavioural decline compared to the placebo (Aisen, 2000). This outcome does not necessarily mean that anti-inflammatory therapy is not useful in AD, as NSAIDs may have favourable effects that are not mediated by steroids. As steroids may cause psychogenic effects, their use in AD to alleviate inflammation may not be appropriate.

Numerous plants and plant constituents have demonstrated anti-inflammatory properties (Bingöl and Şener, 1995; Deepak and Handa, 2000; Handa *et al.*, 1992; Hoult and Payà, 1996; Laughton *et al.*, 1991; Skaltsa *et al.*, 2000; Welton *et al.*, 1986), thus there is potential for novel anti-inflammatory agents to be identified, which may be useful in AD therapy, with fewer adverse effects than drugs currently available. For example, ferulic acid, which is an anti-oxidant and anti-inflammatory compound derived from plants, ameliorated the β-amyloid-induced reduction in ACh (1) levels in the cortex and β-amyloid-induced IL-1 immunoreactivity in the hippocampus in mice, and also improved cognitive function (Yan *et al.*, 2001).

1.2.4 Anti-Oxidant Therapy

Free radicals may damage biological materials, including carbohydrates, DNA, lipids and proteins. The peroxidation of phospholipid cell membranes is a free radical-mediated process. A primary free radical interacts with a polyunsaturated fatty acid

(PUFA), which then rearranges to form a conjugated diene, which may react with O_2 to yield a peroxyl radical. The peroxyl radical can abstract H from another lipid molecule in a propagative process, which initiates a complex series of reactions that yield various degradation products (Halliwell and Gutteridge, 1995; Slater, 1984). The degradation products (e.g. hydroxyalkenals) from lipid peroxidation may cause various biological effects, including enzyme inhibition and modulation of DNA synthesis (Slater, 1984). Lipid peroxide arising from lipid peroxidation in cells is cytotoxic, causing disruption of cell membranes (Ando *et al.*, 1990); this mechanism has been found to be one of the major biochemical markers of aging.

Free radical reactions are reported to initiate cell injury, and have been implicated in the pathology of many diseases including aging processes, atherosclerosis, ischaemic heart disease and neurodegenerative diseases (Maxwell, 1995; Slater, 1984; Spiteller, 1993). It has been proposed that oxidative processes may be involved in AD pathogenesis. Therefore, the use of anti-oxidants may slow AD progression and minimise neuronal degeneration.

1.2.4.1 Oxidative Stress in Alzheimer's Disease

Oxidative damage to proteins and DNA has been found in the brains of AD patients (Lyras *et al.*, 1997). Lipid peroxidation in the brain may be associated with normal aging. In rats, aged brains showed higher levels of lipid peroxides, perhaps due to altered protection systems against oxidative damage (Ando *et al.*, 1990). However, lipid peroxidation is reported to be more extensive in AD brains than normal aged brains. Lipid peroxidation of membranes has been identified in various brain regions, including the hippocampus and amygdala of AD patients and, ApoE peroxidation has been correlated to AD (Balázs and Leon, 1994; Christen, 2000; Lovell *et al.*, 1995), which may reflect the extent of oxidative damage at various stages of AD pathogenesis. Elevated levels of glutathione peroxidase, superoxide dismutase (SOD), glutathione reductase and catalase have also been reported to occur in AD brains, perhaps as a compensatory response to free-radical activity (Lovell *et al.*, 1995).

Most lipid peroxidation *in vivo* is metal-ion dependent (Halliwell *et al.*, 1989). Elevated levels of iron in AD brains have been associated with senile plaques, and this has also been implicated in oxidative processes (Christen, 2000; Connor *et al.*,

1992; Smith *et al.*, 1997). Cerebral ischaemia, which has been implicated in dementia pathology, has also been reported to initiate oxygen radical mechanisms (Traystman *et al.*, 1991).

1.2.4.2 β-Amyloid

Although oxidative processes in AD are well characterised, the source of oxidative stress in AD pathogenesis is unclear. One contributing factor may be β-amyloid. Cortical neurons from Down's syndrome brain, which also show elevated levels of β-amyloid, showed an increase in intracellular levels of reactive oxygen species compared to controls *in vitro*, and spontaneous apoptosis was reduced by free radical scavengers (Buscuglio and Yankner, 1995; Oyama *et al.*, 1994). β-Amyloid inhibited the electrical stimulation of NA release and increased intracellular Ca^{2+} levels in rat brain cortical slices and synaptosomes, respectively; both effects were prevented by N-tert-butyl-α-phenylnitrone (PBN), a free radical scavenger (Li and Smith, 1996). The preventative effect of the free radical scavenger, indicates that free radical generation may be involved in the neurotoxic effects of β-amyloid.

It has been shown that β-amyloid causes oxidative stress to neurons *in vitro* and that free radical attack of cell membranes initiates lipid peroxidation (Behl *et al.*, 1994; Hensley *et al.*, 1994; Mark *et al*, 1995; Marx, 1996). Generation of free radicals, via an unknown mechanism, also promoted the toxicity of β-amyloid in rat hippocampal cells *in vitro* (Manelli and Puttfarcken, 1995). β-Amyloid has also been reported to cause an accumulation of H_2O_2 and lipid peroxides in cells, which may be inhibited by catalase (Behl *et al.*, 1994). These results indicate that β-amyloid may promote oxidative damage to neurons in AD, perhaps due to H_2O_2 via hydroxyl radicals ($^{\bullet}OH$), although other mechanisms of oxidative damage may also occur.

1.2.4.3 Neurofibrillary Tangles

The formation of neurofibrillary tangles has been proposed to occur as a result of oxidative mechanisms, but may also contribute to oxidative stress. An increase in oxidative stress in neurons has been proposed to decrease tau binding to microtubules causing microtubule instability; such events may cause inappropriate tau accumulation and subsequent hyperphosphorylation and facilitation of filament

formation (Guttman *et al.*, 1995; Schweers *et al.*, 1995; Wille *et al.*, 1992). However, treatment of cortical neurons with H_2O_2 did not cause hyperphosphorylation of tau *in vitro* (Davis *et al.*, 1997), indicating that oxidative stress may not cause neurofibrillary tangles. The formation of paired helical filaments containing abnormally hyperphosphorylated tau has been associated with oxidative stress (Johnson and Jenkins, 1996), which may contribute to further oxidative damage to neurons. It remains unclear whether neurofibrillary tangle formation involves oxidative processes *in vivo.*

1.2.4.4 Anti-Oxidants

Oxidative processes appear to be a contributing factor to neuronal degeneration in AD pathogenesis, but their role in the cause of AD remains unclear. The use of anti-oxidants as part of AD therapy may slow deterioration caused by oxidative reactions. Various anti-oxidants have been investigated for their potential in AD therapy. The anti-oxidants vitamin E and propyl gallate (PpG) (**135**) have been shown to protect neurons from β-amyloid toxicity *in vitro* (Behl *et al.*, 1992). An anti-oxidant derivative synthesised from prostaglandin B_2 and ascorbic acid, inhibited free radical formation and lipid peroxidation in rat brain, following ischaemic injury (Sakamoto *et al.*, 1991) and chronic anti-oxidant treatment improved cognitive function in aged rats (Socci *et al.*, 1995).

It is important that anti-oxidants used as part of AD therapy, are able to cross the BBB and reach their site of action. The pyrrolopyrimidine anti-oxidants (e.g. U-101033E) are reported to cross the BBB and enter the brain and are neuroprotective (Schmid-Elsaesser *et al.*, 1997), so may have potential in AD.

Injury of plant cells, as well as mammalian cells, is associated with the activation of lipoxygenases, which catalyse the formation of hydroperoxides of PUFAs; a hydroperoxide radical may react with fatty acids to produce dioxoenes, which are regarded as plant defence compounds (Spiteller, 1993). The occurrence of oxidative mechanisms in plants may explain why an abundance of anti-oxidant compounds have been identified in plant tissue. Their anti-oxidant effects in plants may therefore have relevance in mammals, particularly in disorders involving oxidative stress such as AD.

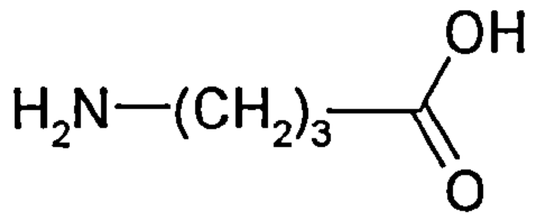
A wide range of plants and their constituents have been shown to be potent anti-oxidants by various mechanisms, including free radical scavenging activity and inhibition of lipid peroxidation.

Some plants with anti-oxidant activity include *Aeseclus hippocastanum*, *Allium mutans*, *Artemisia* spp., *Guiera senegalensis*, *Hamamelis virginiana*, *Rosmarinus officinalis*, *Salvia officinalis*, *Taraxacum officinale* and *Thymus vulgaris* (Aruoma *et al.*, 1996; Bouchet *et al.*, 1998; Cuvelier *et al.*, 1996; Deans *et al.*, 1993; Hagymási *et al.*, 2000; Kimura *et al.*, 1985b; Masaki *et al.*, 1995; Štajner *et al.*, 1999; Youdim and Deans, 1999). Phytochemicals with anti-oxidant effects include cinnamic acids, coumarins, diterpenes, flavonoids, lignans, monoterpenes, phenylpropanoids, tannins and triterpenes (Aesbach *et al.*, 1994; Bouchet *et al.*, 1998; Craig, 1999; Foti *et al.*, 1996; Kelm *et al.*, 2000; Kimura *et al.*, 1985b; Laughton *et al.*, 1989; Lu and Liu, 1991; Okuda *et al.*, 1983; Ruberto and Baratta, 2000; Sassa *et al.*, 1990; Schwarz and Ternes, 1992; Seidel *et al.*, 2000; Youdim and Deans, 2000; Zhu *et al.*, 1999).

Some plants and plant compounds have shown favourable effects in the CNS, so may be appropriate for use in AD. Curcumin from *Curcuma longa* reduced lipid peroxidation in rat brain following oral administration to rats with ethanol-induced brain injury (Rajakrishnan *et al.*, 1999), and *Bacopa monniera*, which is reported to have cognition enhancing effects, induced a dose related increase in SOD, catalase and glutathione peroxidase activities in the rat frontal cortex, striatum and hippocampus (Bhattacharya *et al.*, 2000c). *Thymus vulgaris* essential oil maintained higher PUFA levels in various tissues, including the brain in rats, indicating protective anti-oxidant effects (Youdim and Deans, 1999). Therefore the use of plants and their constituents in the diet and as supplements may be relevant in slowing AD progression.

1.2.5 Modulation of GABAergic Function

GABAergic neurons have been identified in the mammalian nervous system, including the autonomic nervous system and the CNS, and GABA (**16**) is an important inhibitory neurotransmitter within the CNS (Bowery *et al.*, 1981; Bowery *et al.*, 1987; Ong and Kerr, 1983; Parkman *et al.*, 1993; Sivilotti and Nistri, 1991; Wilkin, 1981). GABA (**16**) activates membrane-bound receptors $GABA_A$, $GABA_B$ and $GABA_C$, although other receptor subtypes may also exist.



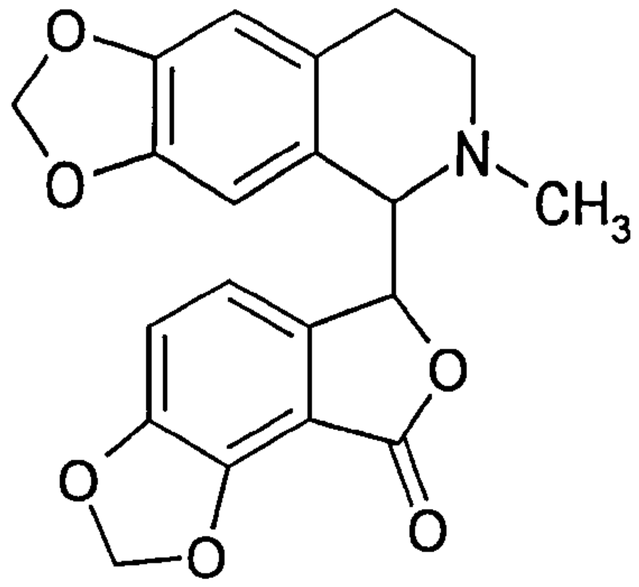
γ-aminobutyric acid (**16**)

1.2.5.1 $GABA_C$ Receptors

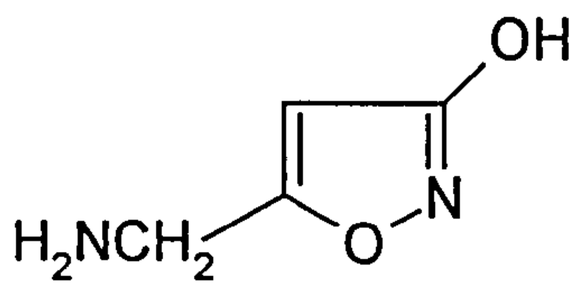
$GABA_C$ receptors have low affinity for $GABA_A$ and $GABA_B$ receptor modulators, including bicuculline (**17**) and baclofen (**20**) respectively (Woodward *et al.*, 1992; Woodward *et al.*, 1993). The role of $GABA_C$ receptors is uncertain but their localisation in retinal neurons suggests that they may be involved in light responses (Feigenspan and Bormann, 1994).

1.2.5.2 $GABA_A$ Receptors and Cognitive Function

$GABA_A$ receptors are involved in the action of anxiolytics (e.g. benzodiazepines) and hypnotics (e.g. barbiturates). The activation of $GABA_A$ receptors by GABA (**16**), which increases neuronal Cl^- conduction, is enhanced by these compounds. Several drugs used for management of epilepsy also influence $GABA_A$ receptors; these include benzodiazepines, valproate and vigabatrin, which enhance GABA-mediated neuronal inhibition. $GABA_A$ receptor ligands have been implicated in cognition. $GABA_A$ agonists disrupt memory in animals and humans and benzodiazepine agonists and inverse agonists impair and enhance cognition respectively, whilst $GABA_A$ antagonists enhance memory (Brioni *et al.*, 1989; Brioni *et al.*, 1990; Izquierdo and Medina, 1991; Ohno *et al.*, 1992; Venault *et al.*, 1986).



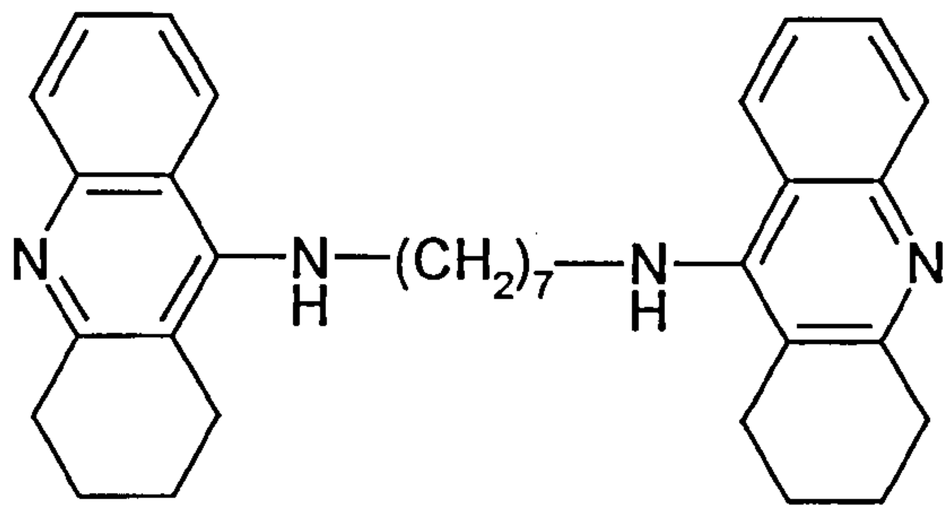
Bicuculline (**17**)



Muscimol (**18**)

Administration of the $GABA_A$ agonist muscimol (**18**) decreased memory acquisition of passive avoidance learning in mice, and also inhibited the cognitive enhancement induced by the AChE inhibitor physostigmine (**5**) (Zarrindast *et al.*, 1998).

The benzodiazepine antagonist ZK 93426 increased cortical ACh (**1**) release in both young and aged rats, and the $GABA_A$ antagonist bicuculline (**17**) increased striatal ACh (**1**) output in rats but, the $GABA_A$ agonist muscimol (**18**) decreased striatal and hippocampal ACh (**1**) release (DeBoer and Westerink, 1994; Gorman *et al.*, 1994; Moore *et al.*, 1992). This suggests that memory enhancing effects of $GABA_A$ receptor modulators may be due to stimulation of cholinergic neurotransmission. The AChE inhibitor bis(7)-tacrine (**19**) is also a potent $GABA_A$ receptor antagonist (Li *et al.*, 1999), which may result in superior cognitive enhancement when compared to $GABA_A$ antagonists or AChE inhibitors alone. However therapeutic use of $GABA_A$ antagonists may induce seizures, therefore the identification of antagonists with partial agonist activity, which also stimulate ACh (**1**) release, may be more appropriate.



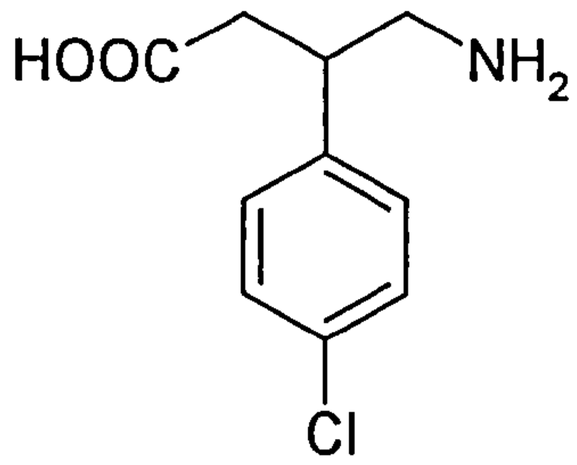
Bis(7)-tacrine (**19**)

The anaesthetics propofol and thiopental have been found to be neuroprotective against anoxia and physical trauma to dendrites, an effect mediated by potentiation of $GABA_A$ receptor function, and the $GABA_A$ agonist muscimol (**18**) was similarly neuroprotective (Hollrigel *et al.*, 1996). Regulation of GABA (**16**) within the CNS may also be neuroprotective by maintaining a balance with glutamate activity. These effects may be clinically relevant in neurodegenerative disorders but, as $GABA_A$ agonists have been reported to impair cognitive function (Brioni *et al.*, 1990), their use in AD management may be unwise and use may be more appropriate in disorders such as head injury.

1.2.5.3 $GABA_B$ Receptors and Cognitive Function

$GABA_B$ receptor ligands are used therapeutically for various disorders. For example, the $GABA_B$ agonist baclofen (**20**) is used to manage spasticity and trigeminal neuralgia, but adverse effects include drowsiness, dizziness, seizures, hallucinations and mental confusion (British National Formulary, 2001). $GABA_B$ receptors, which have been identified in the mammalian CNS, have also been shown to influence sleep regulation, interactions with alcohol and have been implicated in cognition (Allan and Harris, 1989; Crunelli *et al.*, 1992; Getova *et al.*, 1997; Hill and Bowery, 1981; Mondadori *et al.*, 1992).

$GABA_B$ agonists (e.g. baclofen (**20**)) are associated with cognitive impairment (Carletti *et al.*, 1993; Castellano *et al.*, 1989; McNamara and Skelton, 1996; Tang and Hasselmo, 1996), and reduced GABA (**16**) levels and abnormalities in $GABA_B$ receptor systems have been identified in AD (Chu *et al.*, 1987; Klunk *et al.*, 1994; Klunk *et al.*, 1995; Ossowska, 1993). It is therefore feasible that modulation of $GABA_B$ receptors may be of clinical relevance in CNS disorders involving cognitive dysfunction, such as AD.

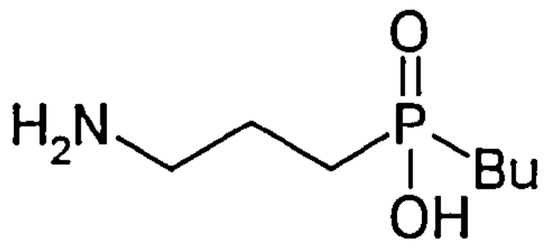


Baclofen (**20**)

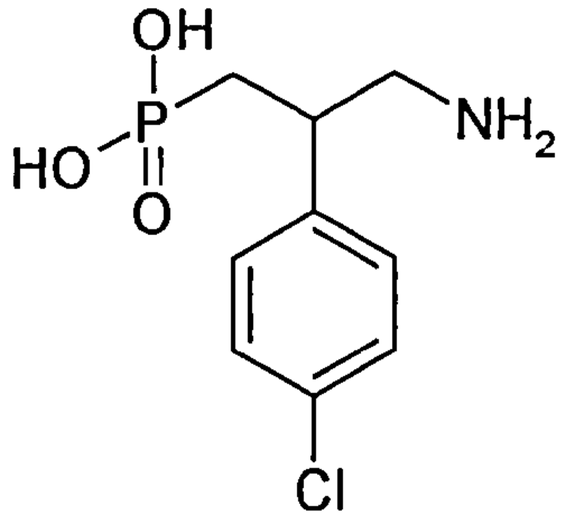
$GABA_B$ presynaptic and postsynaptic receptors differ in their physiological role, and in their affinity for ligands. Antagonism of presynaptic $GABA_B$ receptors is proposed to counteract the autoinhibition of GABA (**16**) release, making the occurrence of long-term potentiation (LTP) more difficult, whereas antagonism of postsynaptic $GABA_B$ receptors is reported to facilitate the occurrence of LTP, and postsynaptic receptors are completely blocked by 100μM CGP 35348, whereas presynaptic receptors require 1mM for complete antagonism (Davies *et al.*, 1991; Olpe *et al.*, 1993). Administration of CGP 35348 to rats enhanced memory moderately at low doses (12.5mg/kg - 25mg/kg), substantially at an intermediate dose (50mg/kg), but at

higher doses (100mg/kg - 300mg/kg) effects on memory declined (Stäubli *et al.*, 1999). This may be explained by memory enhancement (and LTP occurrence) at concentrations at which postsynaptic blockade dominates, but as the concentration of CGP 35348 was increased, antagonism of presynaptic receptors occurred and memory was not facilitated. It is therefore the antagonism of the postsynaptic receptors that may be clinically relevant in cognitive dysfunction.

$GABA_B$ receptor antagonists have been investigated for their potential as anti-amnesic agents. The $GABA_B$ antagonist CGP 36742 (**21**), a compound able to cross the BBB, reverses age-related deficits in old rats in an active avoidance test, and enhances spatial learning performance and antagonises amnesia induced by the $GABA_B$ agonist baclofen (**20**) in mice (Carletti *et al.*, 1993; Froestl *et al.*, 1995). The memory enhancing effects of CGP 36742 (**21**) were detectable only 20hr after a single treatment and although cognition declined with time, effects were still evident after four months, suggesting that CGP 36742 (**21**) may influence long-term memory (Mondadori *et al.*, 1994; Mondadori *et al.*, 1996).



CGP 36742 (**21**)



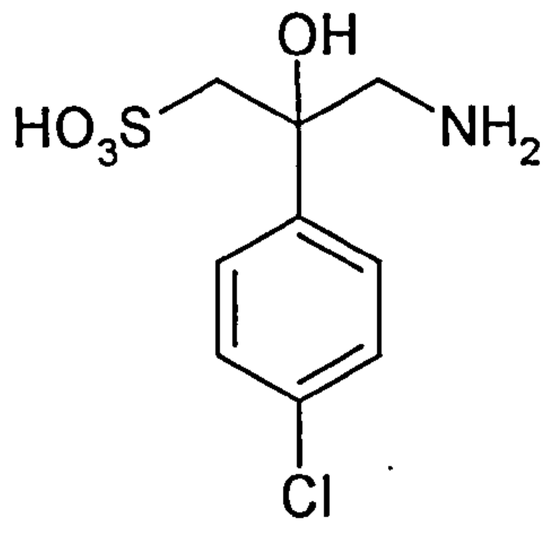
Phaclofen (**22**)

Treatment with CGP 36742 (**21**) also improved learning and memory impairment in mice caused by colchicine, a compound that induces neurofibrillary degeneration, hippocampal lesions and reduces ChAT activity (Bensimon and Chermat, 1991; Nakagawa *et al.*, 1987; Yu *et al.*, 1997). The more potent $GABA_B$ antagonists, CGP 71982, CGP 62349 and CGP 55845A, improved learning and memory retention in active avoidance tests in rats (Getova and Bowery, 1998). These results provide further evidence for the potential of $GABA_B$ antagonists in the management of memory disorders but the mechanism of action of cognition enhancement remains to be established.

The influence of GABA receptor modulation on the cholinergic system has been implicated to explain the effects of GABA agonists and antagonists on cognitive function (Decker and McGaugh, 1991). The $GABA_B$ receptor antagonist phaclofen (**22**) modulates ACh (**1**) release in rat brain slices, the muscarinic receptor agonist oxotremorine attenuates the cognitive impairment induced by baclofen (**20**) in passive avoidance tests in rodents, oral administration of CGP 36742 (**21**) attenuates the baclofen- and scopolamine-induced learning deficit in rats and, baclofen (**20**) decreases the learning improvement induced by physostigmine (**5**) in mice (Arenas *et al.*, 1990; Castellano and McGaugh, 1991; Nakagawa and Takashima, 1997; Zarrindast *et al.*, 1998). CGP 35348 dose-dependently potentiates the excitatory responses of cortical neurons elicited by ACh (**1**) (Andre *et al.*, 1992). The mechanisms for these effects remain to be elucidated but these results indicate that $GABA_B$ receptors may influence cholinergic neurons.

It has also been reported that spontaneous inhibitory postsynaptic currents in the basal forebrain (a major source of cholinergic input to the cerebral cortex) are regulated by $GABA_B$ receptors, and the amygdala and hippocampus, brain areas involved in memory formation, contain cholinergic neurons under the inhibitory control of the GABAergic system (Akaike *et al.*, 1992; Izquierdo and Medina, 1991). The results from these studies suggest that the cognitive enhancement by $GABA_B$ receptor antagonists may involve cholinergic function, although other mechanisms of action may also occur.

Modulation of glutamate release has also been proposed to explain the effects of $GABA_B$ antagonists on cognitive function; an increase in glutamate release may occur following a decrease in GABA-mediated inhibition (Bernasconi *et al.*, 1992; Bernasconi *et al.*, 1993). Muscimol (**18**) and baclofen (**20**) ($GABA_A$ and $GABA_B$ agonists respectively) decrease striatal ACh (**1**) output in rats, the $GABA_A$ antagonist bicuculline (**17**) increases striatal ACh (**1**) release, but the $GABA_B$ antagonist 2-hydroxy-saclofen (**23**) does not influence striatal ACh (**1**) output (DeBoer and Westerink, 1994). It was therefore proposed that although modulation of $GABA_A$ receptors on cholinergic neurons may regulate ACh (**1**) release, the $GABA_B$ mediated effects may be due to presynaptic inhibition of the glutamatergic input of striatal cholinergic neurons. But, it cannot be excluded that $GABA_B$ antagonists other than 2-hydroxy-saclofen (**23**) may enhance ACh (**1**) release.



2-Hydroxy-saclofen (**23**)

$GABA_B$ antagonists are also reported to up-regulate GABA receptors (Pratt and Bowery, 1993), which may also explain the improvements observed in cognitive function. For AD management the use of multiple drug therapy to target neurotransmitter systems involving GABAergic and cholinergic function, may provide clinical advantages over current approaches to enhance cholinergic function alone with AChE inhibitors.

Depression may also be a feature of AD (Berg, and Morris, 1990) but antidepressant use may be limited due to adverse effects (e.g. tricyclic antidepressants are anticholinergic and may impair cognition). $GABA_B$ receptor activation is reported to suppress catecholamine and 5-HT release from brain slices and it was proposed that $GABA_B$ antagonists may alleviate depression by inhibiting the suppression of NA release, induced by GABA (**16**) acting on presynaptic $GABA_B$ receptors on noradrenergic terminals (Bowery *et al*, 1980; Pratt and Bowery, 1993). However, there have been conflicting reports regarding the effect of antidepressants on GABA binding to $GABA_B$ receptors (Arranz *et al*, 1992; Lloyd and Pilc, 1984; Pratt and Bowery, 1993). In view of these findings, the potential for $GABA_B$ antagonists to treat depression cannot be ignored, but does require further investigation.

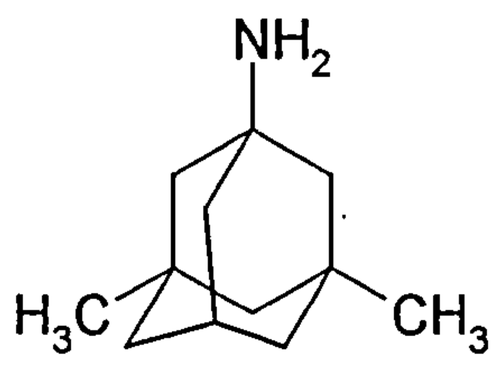
1.2.6 Other Therapeutic Strategies

1.2.6.1 Modulation of Other Neurotransmitter Systems

As several neurotransmitter systems are affected in AD pathogenesis, drugs that enhance neurotransmission, other than cholinergic neurotransmission, have been investigated as potential therapy. Potentiation of noradrenergic function has been

explored, but the α-adrenoceptor agonist clonidine did not show any cognitive benefits (Mohr *et al.*, 1989). The MAO-B inhibitor selegeline reduces dopamine metabolism, so may enhance dopaminergic activity in the brain. Selegeline showed some beneficial effects on behaviour and cognition in AD patients (Schneider *et al.*, 1991; Tariot *et al.*, 1987) but the MAO-B inhibitor milacemide was ineffective in AD patients (Dysken *et al.*, 1992). Enhancement of 5-HT levels using trazodone has been shown to improve the behavioural problems associated with AD (e.g. irritability, restlessness and anxiety) (Lake and Grossberg, 1987; Lebert *et al.*, 1994; Simpson and Foster, 1986). It is apparent that attempts to modulate other neurotransmitter systems to date have been relatively unsuccessful, which emphasises the need to identify alternatives to treat symptoms of AD.

Glutamate may induce neuronal degeneration, by over-stimulation of NMDA receptors. The development of NMDA receptor antagonists may offer some protection against neuronal degeneration in AD. Memantine (**24**), an NMDA receptor antagonist, is reported to be under investigation in phase III clinical trials in the USA and is already approved for use in dementia in Germany (Evans, 2001; Marx, 2000).



Memantine (**24**)

1.2.6.2 Cerebral Vasodilators

Brain capillaries are also affected in AD. β-Amyloid surrounds the brain vasculature, which may result in thickened capillaries with impaired elasticity resulting in impaired cerebral blood flow and eventually degeneration (Vinters *et al.*, 1994).

Cerebral vasodilators, including co-dergocrine, isoxsuprine, cyclandelate and ergoloid mesylates, have been investigated (alone or in combination with lecithin) to enhance cerebral blood flow, but have also shown little promise as potential AD therapy (Jenike *et al.*, 1986; Weyer *et al.*, 2000).

Extracts from *Ginkgo biloba* leaf have improved symptoms in AD patients (Kanowski *et al.*, 1997; Le Bars *et al.*, 1997; Le Bars *et al.*, 2000; Rigney *et al.*, 1999), which was

perhaps due to vasodilatory flavonoids but other mechanisms of action may also be responsible for the favourable effects observed (refer to 1.3, Table 1.1).

1.2.6.3 Modulation of Senile Plaque Formation

Manipulation of senile plaque formation, a critical event in AD pathogenesis, is another approach to AD management. For example, the inhibition of secretases thought to be responsible for APP metabolism, in an attempt to reduce β-amyloid plaque formation, has been reported (Evans, 2001; Kisilevsky, 1998). The compounds brefeldin A and monensin are reported to inhibit the conversion of the immature form of β-secretase (beta-site APP cleaving enzyme (BACE)) from the mature form of BACE, which is under the action of a furin-like proprotein convertase (Bennett *et al.*, 2000). Compounds that bind to presenilin, which form a complex with γ-secretase, are also under investigation for their inhibition of senile plaque formation (Evans, 2001).

Other targets may include reducing β-amyloid deposition, perhaps by enhancing its clearance. The anti-psychotics haloperidol and droperidol dose-dependently inhibited β-amyloid formation in human neurons *in vitro*, which may have occurred via inhibition of proteolysis (Higaki *et al.*, 1997). These findings indicate that some anti-psychotics may have therapeutic potential in AD.

Some proteins are able to bind to β-amyloid, including AChE, which has been shown to accelerate the formation of β-amyloid deposits, and AChE has been found to be localised in senile plaques and also in neurofibrillary tangles (Coleman *et al.*, 1992; Inestrosa *et al.*, 1996a, Inestrosa *et al.*, 1996b; Morán *et al.*, 1993). These findings indicate that AChE may participate in senile plaque formation, and that its inhibition may inhibit senile plaque formation. AChE inhibitors (e.g. tacrine (7)) have been shown to reduce APP secretion *in vitro* (Lahiri *et al.*, 1994; Lahari *et al.*, 2000), suggesting that AChE inhibitors may inhibit senile plaque formation in AD. Transgenic mice that had overexpressed human AChE neurons showed a progressive cognitive decline (Beeri *et al.*, 1995). AChE may impair cognition, perhaps by hydrolysing ACh (1) and affecting neurotransmission, by modulating β-amyloid deposition, by modulating neurofibrillary tangle formation or, by unknown mechanisms.

The prevention of senile plaque formation using a vaccine has also been investigated. The vaccine composed of AN-1792, an artificial form of β-amyloid, was found to reduce existing senile plaques and prevent further senile plaque formation in transgenic mice, and did not stimulate autoimmunity (Helmuth, 2000; Marwick, 2000).

1.2.6.4 Nerve Growth Factor

NGF is important in growth and maintenance of cholinergic neurons and its administration to rodents, improved learning and retention (Fischer *et al.*, 1987; Ricceri *et al.*, 1996; Schneider, 1996). The mechanism to explain these observations has not been established, but could be due to one or more effects. NGF is reported to upregulate cholinergic neuron function, reverse cholinergic neuron atrophy, increase nicotinic receptors, improve cerebral blood flow, raise ChAT levels in forebrain cholinergic neurons *in vitro* and *in vivo* and protect against neuronal degeneration (Fischer *et al.*, 1987; Nordberg, 1996; Sofroniew, 1996). NGF treatment may alleviate symptoms of AD, but may not halt disease progression and potential adverse effects *in vivo* require thorough investigation (e.g. sustained hyperalgesia has been reported to occur (Olsen, 1997; Sofroniew, 1996)). Other problems that may be associated with NGF treatment could be a lack of specificity (for neurons within the CNS) and delivery to the brain would be restricted by the BBB. Direct injection into the brain may be complicated by the potential for infections and perhaps interference in brain activity resulting in convulsions.

Leteprinim potassium (NeoTrofin), which can cross the BBB, is reported to stimulate the synthesis of various growth factors in neuronal cells, and may also enhance μ-secretase activity, which reduces the abnormal metabolism of APP and subsequent senile plaque formation (Evans, 2001).

1.2.6.5 Symptomatic Treatment

Various treatments are also used to relieve symptoms of AD other than memory. The use of anti-psychotics (e.g. thioridazine and haloperidol) for control of agitated behaviour and psychosis is reported to be marginally effective (Schneider *et al.*, 1990; Teri *et al.*, 2000), but their use is limited by extrapyramidal side-effects and anti-

cholinergic effects including dry mouth, constipation, blurred vision and perhaps further impairment of cholinergic function. More effective treatments with fewer adverse effects would be preferred to anti-psychotics.

The use of anxiolytics (e.g. benzodiazepines such as diazepam) is an approach to manage behavioural symptoms and insomnia, but adverse effects on cognition may worsen confusion and cognitive function (British National Formulary, 2001). The use of these drugs should therefore be limited. Other drugs which are reported to be of some use in behavioural disturbances, but not in memory improvement, in AD patients include the 5-HT_{1A} partial agonist buspirone, anticonvulsants (e.g. carbamazipine) and β-blockers (e.g. propranolol) (Blokland, 1996; Kumar *et al.*, 1998b), however side-effects including dizziness and confusion and nausea may make their routine use inappropriate.

Depression, also a feature of AD, warrants the use of anti-depressants (e.g. tricyclics such as imipramine), which should also be used with caution due to the potential cardiovascular and anti-cholinergic effects. Selective serotonin reuptake inhibitors (SSRIs) (e.g. citalopram and sertraline) are reported to be effective in controlling agitated behaviour and depression in AD patients (Kumar *et al.*, 1998b; Nyth and Gottfries, 1996), and may therefore be useful in symptomatic treatment.

1.3 Use of Plants for Management of Cognitive Disorders

There are numerous drugs available in Western medicine which have been directly isolated from plants, or are derived from templates of compounds from plant sources. For example, the cardenolide digoxin, originally from *Digitalis* spp., is used for heart failure and ephedrine, originally from *Ephedra* spp., is used as a decongestant. Phytochemicals that have acted as templates for the development of drugs used clinically, include opioid analgesics (chemically related to opioid compounds from *Papaver somniferum* L.) and the anti-coagulant warfarin (chemically related to the coumarins).

The use of plant extracts for medicinal purposes may offer some benefits over the use of single drugs. For example, the many constituents present in a plant extract may act synergistically to enhance a particular pharmacological activity, may provide more

than one relevant pharmacological activity for a particular disease, or may contain constituents which counteract the toxic effects of other constituents.

Compounds may not only act synergistically with other compounds from the same plant, but may also enhance the activity of compounds from other plant species. This approach has been used in various practices of traditional medicine, including Ayurveda and traditional Japanese and Chinese medicine (TCM) where a mixture of plants is commonly prescribed.

Numerous alkaloids from plant sources have been investigated for their potential in AD therapy, and are now in clinical use (e.g. galantamine (**9**) from *Galanthus nivalis* L. is used in the UK and huperzine A (**10**) from *Huperzia serrata* Thunb. is used in China, for AD). The chemical structure of physostigmine (**5**) from *Physostigma venenosum* Balf., provided a template for the development of rivastigmine (**6**), which is also licensed in the UK for AD. Various other plant species have shown favourable effects in AD, or pharmacological activities indicating the potential for use in AD therapy, some of which are summarised in Table 1.1.

The pharmacological activities of plants and their constituents often reflect the use of the plant in traditional herbal medicine. For example, the traditional Japanese prescription Choto-san, which is composed of eleven crude drugs (e.g. *Ginseng* radix, *Glycyrrhizae* and *Zingiberis* rhizoma), was used traditionally to treat cerebrovascular related disorders; recent studies showed anti-amnesic effects in mice and a clinical trial showed favourable effects in patients with vascular dementia (Terasawa *et al.*, 1998; Yuzurihara *et al.*, 1999).

This ethnopharmacological approach has provided leads to identifying potential new drugs, including those for memory disorders (some of which are summarised in Table 1.1) and may aid the discovery of a more varied and efficacious selection of drugs for AD treatment. In the present study, various plants used in traditional Ayurvedic, Chinese and European medicines for their memory-enhancing and anti-dementia effects, were investigated to identify any pharmacological basis for their reputed activities. The plants selected for investigation are described (refer to 1.4).

Table 1.1. Some plants and isolated compounds with relevant activities in relation to treatment of cognitive disorders, including Alzheimer's disease.

Plant	**Compounds Isolated**	**Traditional Uses, Pharmacological and Clinical Effects**
Angelica archangelica L.	Compounds responsible for displacement of nicotine (**2**) receptor binding are unknown.	The crude alcoholic extract of *Angelica archangelica* displaced nicotine (**2**) binding to nicotine receptors in a concentration dependent manner (Perry *et al.*, 1996).
Artemisia absinthium L.	Compounds responsible for displacement of nicotine (**2**) receptor binding are unknown.	The crude alcoholic extract of *Artemisia absinthium* displaced nicotine (**2**) binding to nicotine receptors in a concentration dependent manner (Perry *et al.*, 1996).
Bacopa monniera L.	Compounds responsible for activity require further study, but activity may be due to bacosides A and B (saponins).	*Bacopa monniera* has been used in Ayurvedic medicine to improve memory and intellect. *Bacopa monniera* extract is reported to facilitate learning acquisition and showed anti-oxidant effects in rat frontal cortex, striatum and hippocampus; bacosides A and B are reported to inhibit the amnesic effects of scopolamine in rodents (Bhattacharya *et al.*, 2000c).

Table 1.1. (Continued) Some plants and isolated compounds with relevant activities in relation to treatment of cognitive disorders, including Alzheimer's disease.

Plant	**Compounds Isolated**	**Traditional Uses, Pharmacological and Clinical Effects**
Biota orientalis L.	Compounds responsible for activity require further study.	*Biota orientalis* is used in TCM for insomnia and amnesia. *Biota orientalis* extract ameliorated the memory acquisition disorder induced by amygdala lesions in mice (Nishiyama *et al.*, 1992).
Coptis chinensis Franch.	Berberine and palmatine (alkaloids).	*Coptis chinensis* and berberine and palmatine are AChE inhibitors (Huang, 1993), suggesting potential in AD therapy.
Crocus sativus L.	Crocin (diterpene).	*Crocus sativus* was used in folk medicine as an antispasmodic, sedative, carminative and nerve sedative. *Crocus sativus* extract and crocin improved ethanol-induced impairment of learning behaviour in mice (Abe and Saito, 2000).
Evodia rutaecarpa Benth.	Dehydroevodiamine (alkaloid).	*Evodia rutaecarpa* is used in TCM for cardiovascular and analgesic effects. *Evodia rutaecarpa* and dehydroevodiamine inhibited AChE *in vitro* in a dose-dependent manner, and reversed scopolamine-induced memory impairment in rats (Park *et al.*, 1996). Dehydroevodiamine is also reported to increase cerebral blood flow (Haji *et al.*, 1994).

Table 1.1. (Continued) Some plants and isolated compounds with relevant activities in relation to treatment of cognitive disorders, including Alzheimer's disease.

Plant	**Compounds Isolated**	**Traditional Uses, Pharmacological and Clinical Effects**
Galanthus nivalis L. (also *Narcissus* spp.)	Galantamine (**9**) (alkaloid).	*Galanthus nivalis* was used traditionally in Bulgaria and Turkey for neurological conditions (Shu, 1998). Galantamine (**9**) slowed the decline in cognition and functional ability in AD patients (Wilcock *et al.*, 2000).
Ginkgo biloba L.	Ginkgolides (e.g. ginkgolide B) (sesquiterpenes and diterpenes) and flavonoids.	*Ginkgo biloba* is used in TCM for respiratory disorders, and its use in circulatory disorders dates back to the 1960s (Kenner and Requena, 1996). The plant extract EGb 761 showed favourable effects on cerebral circulation, on neuronal cell metabolism, on the muscarinic cholinergic system, showed anti-oxidant activity and was neuroprotective against β-amyloid-induced toxicity and NO-induced toxicity *in vitro* (Barth *et al.*, 1991; Bastianetto *et al.*, 2000a; Bastianetto *et al.*, 2000b; Heiss and Zeiler, 1978; Kristofiková *et al.*, 1992; Tea *et al.*, 1987; Yao *et al.*, 2001) (continued on next page).

Table 1.1. (Continued) Some plants and isolated compounds with relevant activities in relation to treatment of cognitive disorders, including Alzheimer's disease.

Plant	**Compounds Isolated**	**Traditional Uses, Pharmacological and Clinical Effects**
Ginkgo biloba L.	Ginkgolides (e.g. ginkgolide B) (sesquiterpenes and diterpenes) and flavonoids.	Ginkgolide B is a platelet-activating factor (PAF) antagonist (Braquet *et al.*, 1994), indicating activity against inflammatory processes. Clinical efficacy (modest improvements in cognitive function) of *Ginkgo biloba* extracts, including EGb 761, was observed following administration to AD and non-AD patients in various studies, including randomised, double-blind, placebo-controlled, multi-centre trials (Kanowski *et al.*, 1997; Le Bars *et al.*, 1997; Le Bars *et al.*, 2000; Rigney *et al.*, 1999).
Huperzia serrata Thunb.	Huperzine A (**10**) (alkaloid).	*Huperzia serrata* is used in TCM for promoting circulation, for fever and as an anti-inflammatory and analgesic, and the prescription, Qian Ceng Ta, has been used in TCM to alleviate problems of memory loss (Foster, 1989; Skolnick, 1997). Huperzine A (**10**) is an AChE inhibitor *in vitro* and *in vivo* (Ashani *et al.*, 1992; Laganière *et al.*, 1991; McKinney *et al.*, 1991; Wang *et al.*, 1986b) (continued on next page).

Table 1.1. (Continued) Some plants and isolated compounds with relevant activities in relation to treatment of cognitive disorders, including Alzheimer's disease.

Plant	**Compounds Isolated**	**Traditional Uses, Pharmacological and Clinical Effects**
Huperzia serrata Thunb.	Huperzine A (**10**) (alkaloid).	Huperzine A (**10**) is reported to decrease neuronal cell death induced by glutamate toxicity (Skolnick, 1997). Huperzine A (**10**) improved memory retention processes in cognitively impaired aged and adult rats (Lu *et al.*, 1988). Huperzine A (**10**) improved cognitive performance in AD patients (Shu, 1998). Huperzine A (**10**) is used in China for the symptomatic treatment of AD (Houghton, 1999; Skolnick, 1997).
Hypericum perforatum L. and *Hypericum calycinum* L.	Hypericin (bianthraquinone) and hyperforin (phenolic phloroglucinol derivative).	*Hypericum perforatum* and *Hypericum calycinum* were found to be as effective as anti-depressant drugs (e.g. desipramine) in animal models (Öztürk *et al.*, 1996).
Lippia stoechadifolia H. B. + K.	Pulegone-1, 2-epoxide (monoterpene).	Pulegone-1, 2-epoxide was shown to inhibit AChE *in vitro* (Grundy and Still, 1985), indicating potential as AD therapy.
Macleaya cordata Willd.	Sanguinarine (alkaloid).	Sanguinarine is an inhibitor of AChE (Huang, 1993), therefore may have potential in AD therapy.

Table 1.1. (Continued) Some plants and isolated compounds with relevant activities in relation to treatment of cognitive disorders, including Alzheimer's disease.

Plant	**Compounds Isolated**	**Traditional Uses, Pharmacological and Clinical Effects**
Magnolia officinalis L.	Honokiol and magnolol (phenolic compounds).	Honokiol and magnolol are reported to increase ChAT activity and inhibit AChE activity *in vitro*, and increased hippocampal ACh (**1**) release *in vivo* (Hou *et al.*, 2000).
Melissa officinalis L.	Compounds responsible for displacement of nicotine (**2**) receptor binding are unknown.	In traditional European medicine, *Melissa officinalis* leaf was acclaimed for restoring memory (McVicar, 1994). The crude alcoholic extract of *Melissa officinalis* displaced nicotine (**2**) binding to nicotine receptors in a concentration dependent manner (Perry *et al.*, 1996) (also refer to 1.4.3.2).
Physostigma venenosum Balf.	Physostigmine (**5**) (alkaloid).	*Physostigma venenosum* was used traditionally in Africa as a ritual poison, claimed to determine the guilt or innocence of persons accused of a crime (McCaleb, 1990). Physostigmine (**5**) is reported to protect mice against cognitive impairment caused by oxygen deficit, and to improve learning in rats (McCaleb, 1990). Physostigmine (**5**) antagonised scopolamine-induced impairment of cognitive function in rats (Yoshida and Suzuki, 1993) (continued on next page).

Table 1.1. (Continued) Some plants and isolated compounds with relevant activities in relation to treatment of cognitive disorders, including Alzheimer's disease.

Plant	**Compounds Isolated**	**Traditional Uses, Pharmacological and Clinical Effects**
Physostigma venenosum Balf.	Physostigmine (**5**) (alkaloid).	Physostigmine (**5**) is reported to have shown significant benefits in cognition in both normal and AD patients (Shu, 1998; Sitaram *et al.*, 1978).
Salvia lavandulaefolia Vahl.	α-Pinene (**53**), 1, 8-cineole (**56**) and camphor (**57**) (monoterpenes).	*Salvia lavandulaefolia* essential oil and the oil constituents α-pinene (**53**), 1, 8-cineole (**56**) and camphor (**57**) inhibited AChE *in vitro* (Perry *et al.*, 2000a).
Salvia officinalis L.	Camphor (**57**), citral, 1, 8-cineole (**56**), linalool (**74**) and α-pinene (**53**) (monoterpenes).	*Salvia officinalis* was used traditionally to 'strengthen' the brain and enhance memory: John Gerard stated that 'Sage is singularly good for the head and brain, it quickeneth the senses and memory (Greive, 1984). *Salvia officinalis* extract inhibited brain AChE in a concentration dependent manner (Perry *et al.*, 1996). Camphor (**57**), citral, 1, 8-cineole (**56**), linalool (**74**) and α-pinene (**53**) (constituents known to be present in *Salvia officinalis* essential oil) inhibited AChE *in vitro* (Perry *et al.*, 2000a; Ryan and Byrne, 1988).

1.4 Plants With Reputed Anti-Dementia or Memory Enhancing Effects Investigated in this Study

In the present study, plants with reputed beneficial activities in relation to AD were selected with reference to traditional Ayurvedic, Chinese and European medicine, and also by consultation with traditional herbal practitioners. The plant parts used in the pharmacological investigations were those used traditionally, for their reputed memory-enhancing or anti-aging effects. The plants selected are discussed below.

1.4.1 Traditional Ayurvedic Medicinal Plants

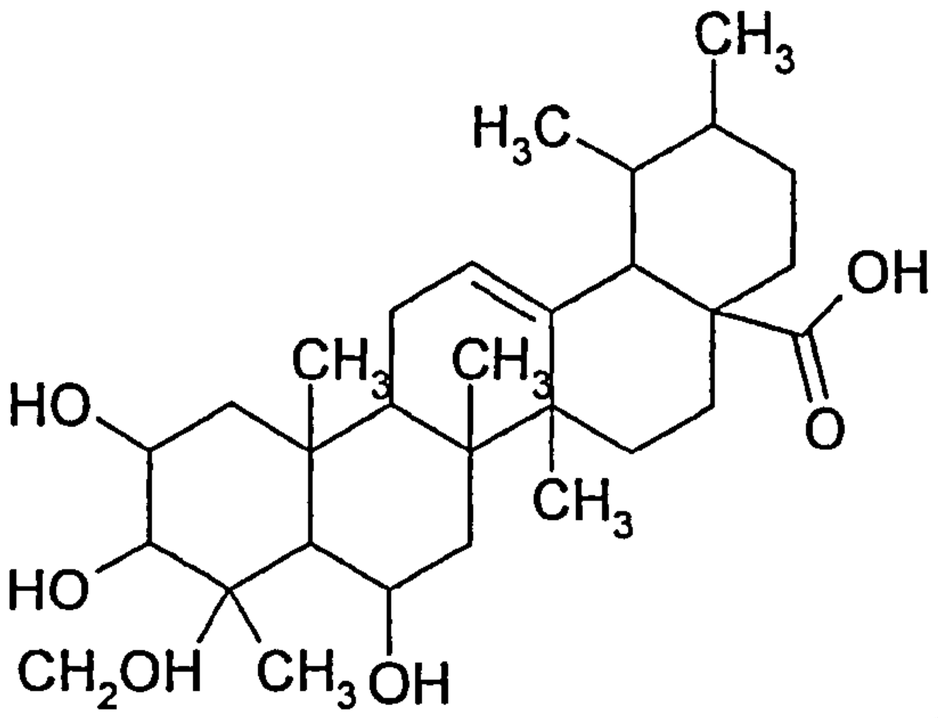
1.4.1.1 *Centella asiatica* L. (Umbelliferae)

Centella asiatica leaf is an ancient Ayurvedic remedy, which is used as a revitalising herb that strengthens nervous function and memory. It is reported to restore youth, memory and longevity (Kapoor, 1990). For example, an Ayurvedic formulation composed of four herbs, including *C. asiatica*, is used to retard age and prevent dementia, and the herb combined with milk is given to improve memory (Manyam, 1999). The herb is also taken as a tonic for poor digestion and rheumatism; the latter suggesting it may have anti-inflammatory effects. *C. asiatica* is also used in TCM for skin ailments and ulcers, as well as leprosy and syphilis and, is used for combating physical and mental exhaustion (Brinkhaus *et al.*, 2000; Duke and Ayensu, 1995).

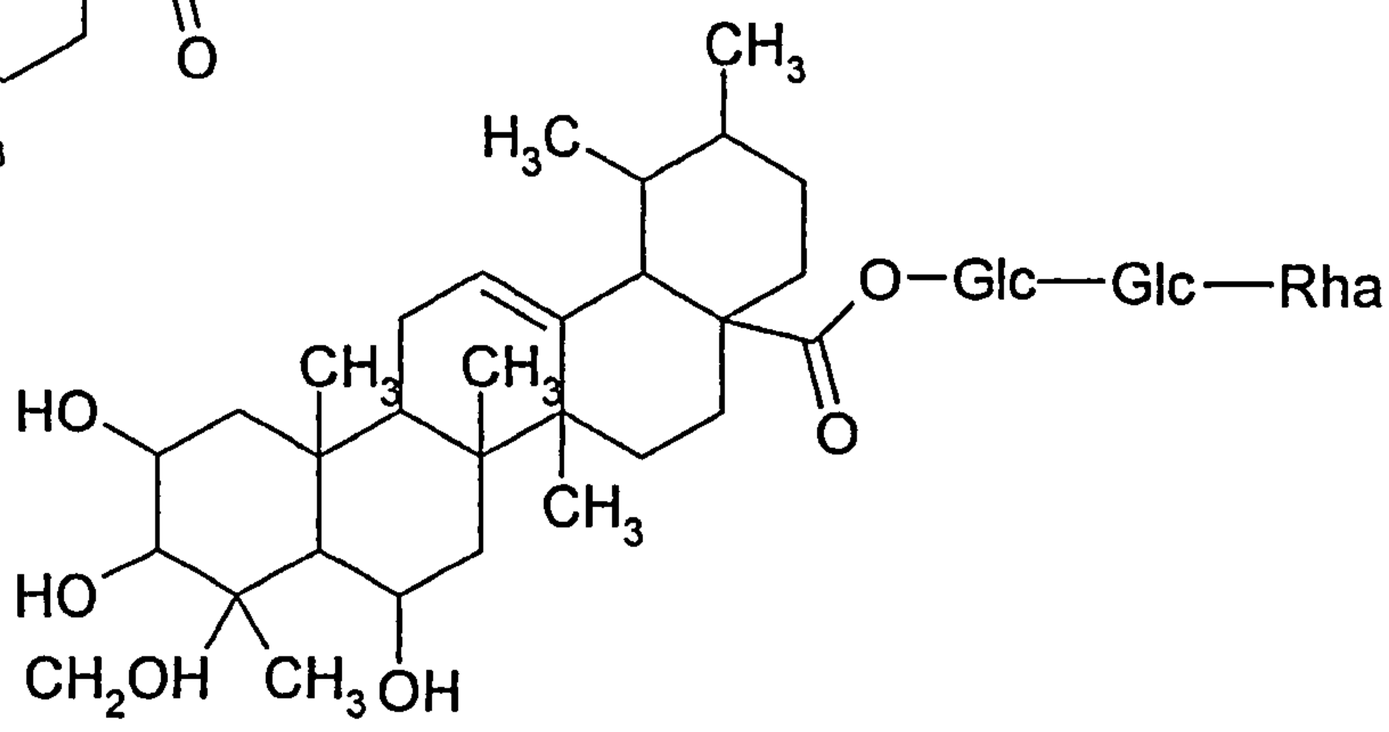
An essential oil (0.1% of the plant), containing monoterpenes and sesquiterpenes, has been extracted from *C. asiatica* leaf (Asakawa *et al.*, 1982; Brinkhaus *et al.*, 2000). The monoterpenes present in the essential oil include bornyl acetate (**51**), myrcene (**52**), α-pinene (**53**), β-pinene (**54**) and γ-terpinene (**55**) and the sesquiterpenes include caryophyllene (**82**), elemene, *trans*-farnesene and germacrene D (Asakawa *et al.*, 1982; Brinkhaus *et al.*, 2000). The triterpenes, which account for 1% - 8% of the constituents isolated, include asiatic acid (**102**), asiaticoside (**101**), brahmoside, brahminoside, madecassic acid (**25**), madecassoside (**26**) and thankuniside (Brinkhaus *et al.*, 2000; Günther and Wagner, 1996; Kapoor, 1990; Maquart *et al.*, 1999).

Various alkaloids have also been isolated from *C. asiatica*, including hydrocotylin (Duke and Ayensu, 1995). A bitter principle vallerine, is present in the roots and leaves, along with amino acids (e.g. alanine, serine), flavonoids (e.g. quercetin (**104**),

kaempferol), fatty acids (e.g. linoleic acid, oleic acid, palmatic acid), phytosterols (e.g. campesterol, sitosterol (**88**), stigmasterol), resin and tannins (Brinkhaus *et al.*, 2000; Duke and Ayensu, 1995; George and Gnanarethinam, 1975; Kapoor, 1990).



Madecassic Acid (**25**)



Madecassoside (**26**)

Although used in Ayurvedic medicine for memory disorders, relatively few studies have been conducted regarding the pharmacological basis of the reputed anti-amnesic effect. An alcoholic extract of the leaves is reported to be tranquillising in rats, an activity that was attributed to brahmoside (Kapoor, 1990; Sakina and Dandiya, 1990). In mice, an extract of *C. asiatica* leaf was sedative, anti-depressant and showed cholinomimetic activity, which was blocked by atropine (Sakina and Dandiya, 1990). These results indicate that *C. asiatica* may be appropriate to treat symptoms of depression and anxiety in AD, and may also enhance cholinergic activity and thus, cognitive function. The mechanism for the potential cholinergic effects of the herb, remains to be established. An aqueous extract of *C. asiatica* leaf improved learning and memory processes in rats, and modulated dopamine, 5-HT and NA systems in rat brain *in vivo* (Nalini *et al.*, 1992). These results suggest that the more polar compounds (e.g. saponins) present in *C. asiatica* leaf may enhance cognitive function by influencing neurotransmitter systems in the CNS. Further studies are necessary to confirm this to identify any potential relevance in AD treatment.

Numerous studies have been conducted on the wound healing effects of *C. asiatica* (Maquart *et al*., 1999; Shukla *et al*., 1999), which reflects its use in Ayurvedic medicine for wound healing following topical application. Other pharmacological activities of *C. asiatica* leaf include anti-bacterial, anti-protozoal and anti-viral activities and anti-convulsant, diuretic, hypoglycaemic and immunomodulatory effects (Brinkhaus *et al*., 2000; Kapoor, 1990).

1.4.1.2 *Withania somnifera* L. (Solanaceae)

Withania somnifera root (ashwagandha) is one of the most highly regarded herbs in Ayurvedic medicine and its use dates back almost 4000 years. It is classed among the rejuvenative tonics ("Rasayanas") and the Ayurvedic scholar Charaka (10BC) wrote about *W. somnifera*, "One obtains longevity, regains youth, gets a sharp memory and intellect and freedom from diseases, gets a lustrous complexion, and strength of a horse" (Upton, 2000). The herb is also traditionally used to treat inflammatory conditions, including bronchitis and arthritis.

Figure 1.1. *Withania somnifera* plant.

Figure 1.2. *Withania somnifera* root.

The primary constituents of the root include numerous alkaloids, which comprise 0.13% - 4.3% of the herb, including anaferine (**60**), anahygrine, ashwagandhine, aswhagandhinine, cuscohygrine (**61**), dl-isopelletierine isopelletierine, pseudotropine, pseudowithanine, somniferiene, somniferinine, tropine (**62**), visamine, withanine,

withaninine and withasomine (Harborne and Baxter, 1993; Mills and Bone, 2000; Schwarting *et al.*, 1963; Upton, 2000). Nicotine (**2**) is reported to be present in *W. somnifera* root (Kapoor, 1990), but some investigations have not detected its presence (Das *et al.*, 1963; Schwarting *et al.*, 1963). This may be explained by variation in the source of the herb, as chemical composition may be influenced by several factors, including environmental conditions for cultivation and storage conditions, or by different extraction procedures used to isolate chemical constituents.

Several steroidal derivatives have also been isolated from *W. somnifera* root. These include the withanolide aglycones (e.g. 5-dehydroxywithanolide R, withanolides A - Y, withasomniferols A - C, withaferin A (**109**), withasomidienone, withasomniferin A) and the glycosylated sitoindosides (e.g. sitoindosides VII - X) (Ghosal *et al.*, 1989; Mishra *et al.*, 2000; Rhaman *et al.*, 1991; Rhaman *et al.*, 1993).

Other constituents reported to be present include amino acids (e.g. aspartic acid, cysteine, glycine), choline, essential and fixed oils, glycosides, phenylpropanoids (e.g. cholorogenic acid (**107**)), phytosterols (e.g. sitosterol (**88**)) and saccharose (Bhattacharya *et al.*, 1987; Ghosal *et al.*, 1988; Kapoor, 1990; Upton, 2000).

There have been numerous studies regarding the cognitive enhancing activities of *W. somnifera*. For example, sitoindosides IX (**65**) and X (**66**) augmented learning acquisition and memory in both young and old rats (Ghosal *et al.*, 1989). The mechanisms for this effect are unclear, but may involve modulation of cholinergic neurotransmission, as an extract containing the sitoindosides VII - X and withaferin A (**109**) was administered to mice and effects on the neurotransmitter systems in the brain were observed. The results showed that the extract enhanced AChE activity in the lateral septum and globus pallidus and decreased AChE activity in the vertical diagonal band, enhanced M_1 receptor binding in the lateral and medial septum and in the frontal cortices, and increased M_2 receptor binding sites in cortical regions but did not affect $GABA_A$, benzodiazepine receptor binding, nor NMDA or AMPA glutamate receptor subtypes (Schliebs *et al.*, 1997). The extract containing the sitoindosides VII - X and withaferin A (**109**) also reversed the ibotenic acid-induced cognitive deficit and reversed the reduction in cholinergic markers (e.g. ACh (**1**), ChAT) in rats (Bhattacharya *et al.*, 1995). The reputed cognition enhancing effects of *W. somnifera* root may therefore be explained by a preferential action on cholinergic neurotransmission in the cortical and basal forebrain, brain areas involved in cognitive

function. These observations indicate that the sitoindosides VII - X and withaferin A (**109**) may have potential in AD therapy.

The methanol extract of *W. somnifera* root dose-dependently promoted dendrite formation in human neuroblastoma cells *in vitro* (Tohda *et al.*, 2000). If this effect occurred in the CNS, treatment of AD patients with the root extract may promote synaptic formation, which involves neurite outgrowth; thus cholinergic function may be enhanced.

The glycowithanolides showed anxiolytic and anti-depressant activities in rats (Bhattacharya *et al.*, 2000d), which may be applicable in the symptomatic treatment of AD. An alkaloid extract also had tranquillising effects *in vivo*, and potentiated barbiturate-, ethanol- and urethane-induced hypnosis in mice (Malhotra *et al.*, 1965).

W. somnifera root and some constituents are also reported to have anti-oxidant and anti-inflammatory activities, which may also be relevant in AD therapy. The crude extract is reported to inhibit lipid peroxidation *in vitro* and *in vivo* (Dhuley *et al.*, 1998; Panda *et al.*, 1997; Panda and Kar., 1998). The root extract and the glycowithanolides (consisting of equimolar concentrations of sitoindosides VII - X and withaferin A (**109**)) are hepatoprotective in rats and mice, an effect attributed to the anti-oxidant activity against hepatic lipid peroxidation (Bhattacharya *et al.*, 2000a; Chaurasia *et al.*, 2000).

The compounds responsible for anti-oxidant activity include the withanolides (Bhattacharya *et al.*, 2001; Mills and Bone, 2000; Mishra *et al.*, 2000; Scartezzini and Speroni, 2000; Upton, 2000), but other compounds in the root may also be anti-oxidant. The glycowithanolides decreased lipid peroxidation in various tissues including the brain in rodents, and both the glycowithanolides and the sitoindosides (VII - X) enhanced catalase and glutathione peroxidase activities in rat frontal cortex and striatum (Bhattacharya *et al.*, 1997; Bhattacharya *et al.*, 2001; Chaurasia *et al.*, 2000).

A root extract was effective against arthritis in rats (Begum and Sadique, 1988) and reduced serum protein levels (e.g. α2-macroglobulin, an indicator of arthritis and inflammatory conditions) (Anbalagan and Sadique, 1981a; Anbalagan and Sadique, 1981b; Anbalagan and Sadique, 1985), and in a double-blind, placebo-controlled, cross-over study, treatment in patients with osteoarthritis resulted in an improvement in symptoms (Kulkani *et al.*, 1991). *W. somnifera* administration to mice treated with a carcinogen reduced IL-1 and TNF-α levels (Dhuley, 1997), which may also be

relevant in AD, considering the possible involvement of these inflammatory mediators in senile plaque formation and neurodegeneration. *W. somnifera* leaves are also reported to have anti-inflammatory activity (Sudhir *et al.*, 1986). The numerous pharmacological activities of *W. somnifera* root indicate that this herb may have multiple beneficial effects in AD patients; this also emphasises the advantages the complex mixture of an extract may have over a pure compound.

W. somnifera root has shown adaptogenic effects (i.e. enhances the resistance of an organism to various stress-inducing effects) *in vivo*. For example, the root extract and sitoindosides IX (**65**) and X (**66**) produced anti-stress activity in mice, assessed by various parameters (e.g. increased physical endurance in swimming) (Archana and Namasivayam, 1999; Dhuley, 2000; Ghosal *et al.*, 1989; Rege *et al.*, 1999; Singh *et al.*, 2000). The herbal formulation Siotone, composed of five herbs including *W. somnifera*, showed adaptogenic activities against a variety of behavioural, biochemical and physiological stress-inducing effects in rats (Bhattacharya *et al.*, 2000b).

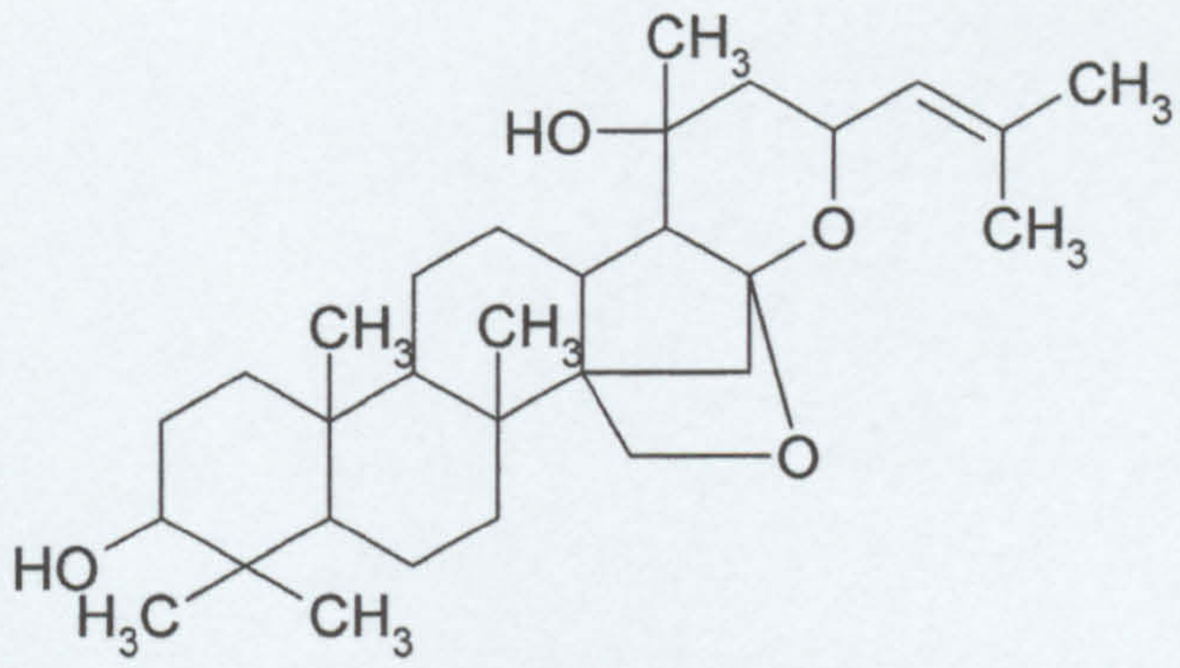
Other reported activities of *W. somnifera* include anti-microbial, anti-tumour, hypocholesterolaemic, hypotensive, diuretic, hypoglycaemic, immunomodulatory and thyroid-stimulating effects (Andallu and Radhika, 2000; Asthana and Raina, 1988; Budhiraja and Sudhir, 1987; Ghosal *et al.*, 1989; Mishra *et al.*, 2000; Panda and Kar, 1999).

1.4.1.3 *Ziziphus jujuba* Mill. (Rhamnaceae)

Ziziphus jujuba is used for numerous conditions in Ayurvedic medicine. The bark is used for fever, gout, rheumatism and to heal ulcers and wounds, the leaves are used as a laxative, the fruit is used for bronchitis, fever and hepatic disorders and the seeds are used for skin disorders and coughs and also as a tonic for the heart and brain (Kapoor, 1990; Kirtikar and Basu, 1935). *Z. jujuba* is also used in TCM: the fruit is used as a sedative and for arthritis, the plant is used to treat nephritis and nervous disease and the seeds are used to treat insomnia, palpitations, anxiety, vertigo and amnesia (Chang and But, 1987; Duke and Ayensu, 1995; Pharmacopoeia of the People's Republic of China, 1992).

Figure 1.3. *Ziziphus jujuba* fruit used in the present study.

The seeds contain 0.1% saponins (e.g. jujubosides A and B, which yield the aglycone jujubogenin (**27**) upon hydrolysis), flavone-*C*-glycosides (e.g. swertisin (**150**) and spinosin (**149**)), triterpenes (e.g. betulic acid and betulin), and a large amount of fixed oil (32%) (Chang and But, 1987; Tang and Eisenbrand, 1992).



Jujubogenin (**27**)

There is a lack of studies aimed at investigating the pharmacological basis for the reputed anti-amnesic effect of *Z. jujuba* seeds. The CNS activities that have been reported to occur *in vivo* include anxiolytic, anti-convulsant, sedative and hypnotic effects (Chang and But, 1987; Peng *et al.*, 2000). Sedative effects have been

attributed to the flavones, particularly swertisin (**150**) (Tang and Eisenbrand, 1992), but other unidentified compounds may also have sedative effects.

Other activities reported to occur with *Z. jujuba* seeds include analgesic, hypothermic and hypotensive effects and seeds were effective as a treatment for burns (Chang and But, 1987).

1.4.2 Traditional Chinese Medicinal Plants

1.4.2.1 *Alisma orientalis* (Sam.) Juzepcz. (Alismaceae)

Alisma orientalis root is used in TCM as a diuretic in the treatment of oedema, and is used in hyperlipidaemia, hypertension, vertigo and acute urinary infection (Chang and But, 1987; Huang, 1993; Pharmacopoeia of the People's Republic of China, 1992; Tang and Eisenbrand, 1992). Constituents isolated from the root include triterpenes (e.g. alisols A (**28**), B (**30**) and C (**32**), alisol A monoacetate (**29**), B monoacetate (**31**) and C monoacetate (**33**), epi-alisol A, alismaketones B 23-acetate and C 23-acetate) and sesquiterpenes (e.g. alismol (**34**), alismoxide (**35**) and orientalols A (**136**), B (**137**) and C (**138**)) (Huang, 1993; Matsuda *et al*., 1999; Murata *et al*., 1970; Murata and Miyamoto, 1970; Oshima *et al*., 1983; Yoshikawa *et al*., 1992).

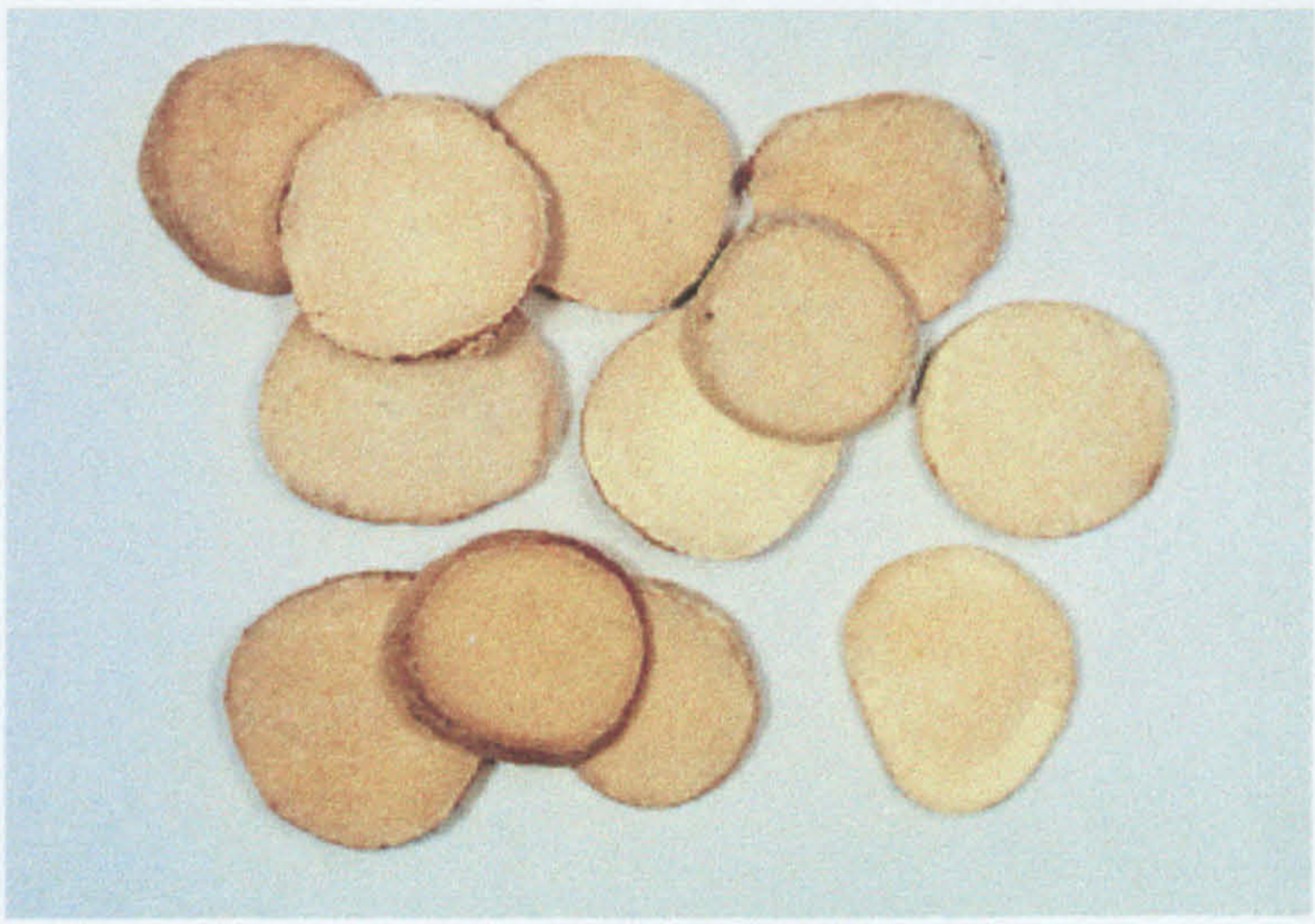
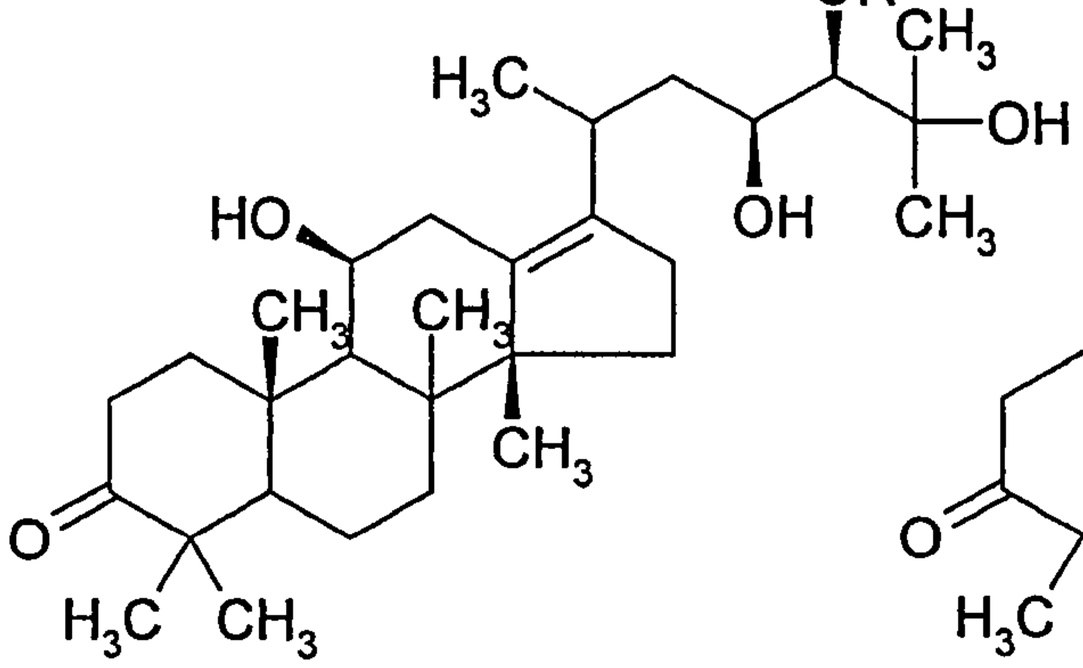
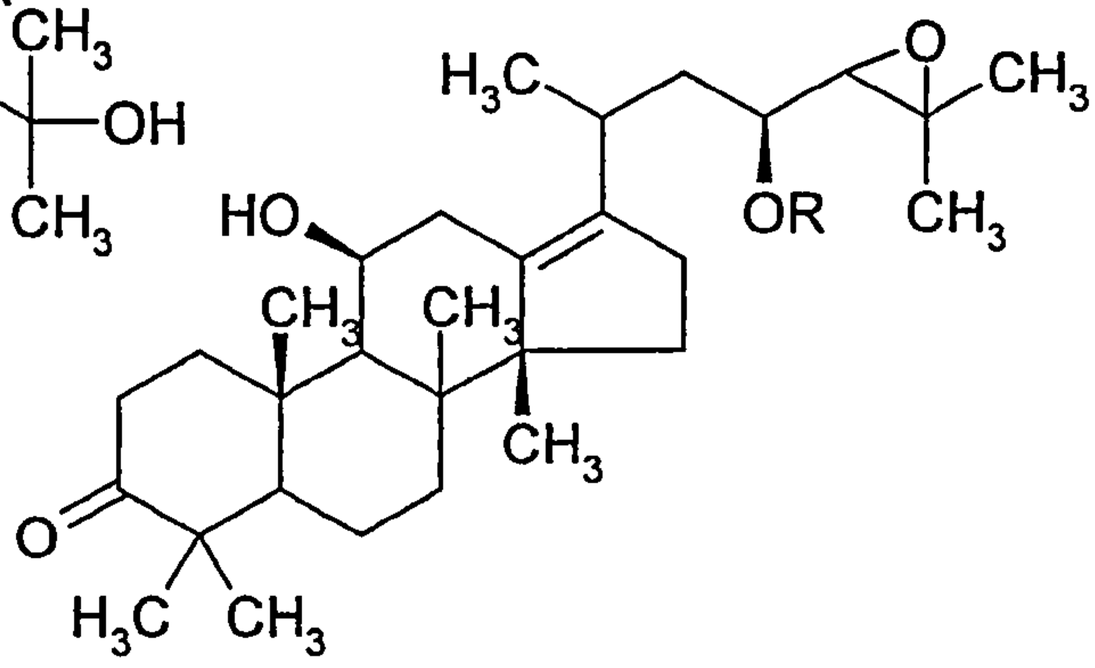


Figure 1.4. *Alisma orientalis* root used in the present study.

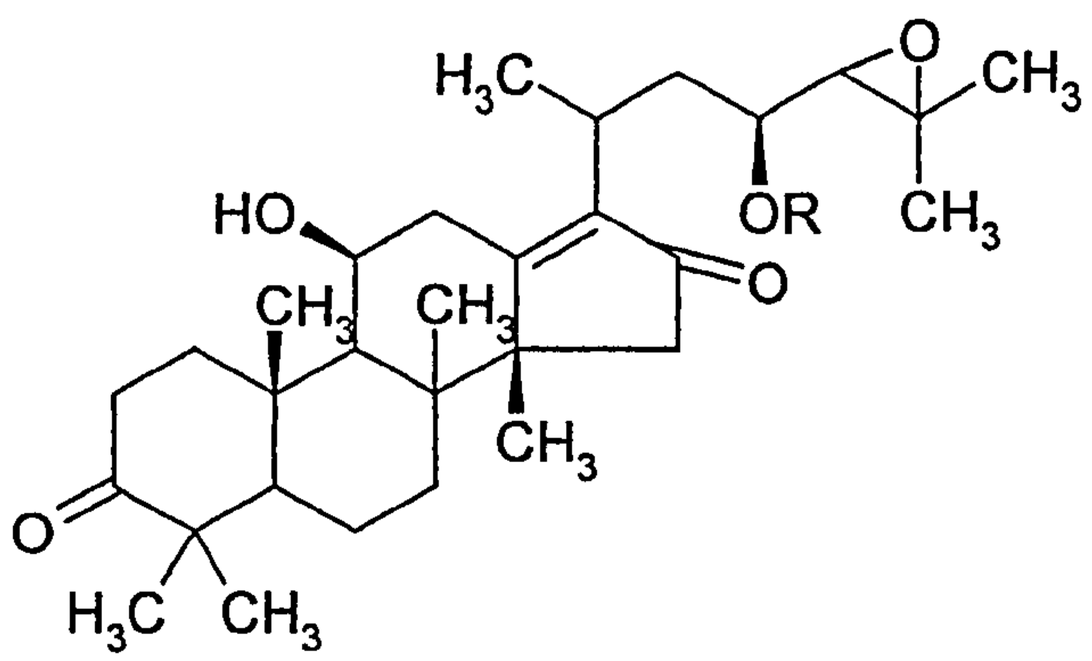
The root is also reported to contain alkaloids, amino acids (e.g. valine), choline, lecithin, essential oil, stigmasterol and sugars (e.g. fructose, glucose, sucrose) and large quantities of starch (Chang and But, 1987; Huang, 1993; Shimizu *et al.*, 1994; Tomoda *et al.*, 1994). It is improbable that the presence of choline and lecithin in the root can explain reputed beneficial effects on memory as these compounds have not been shown to significantly improve cognitive function (Kumar *et al.*, 1998b).



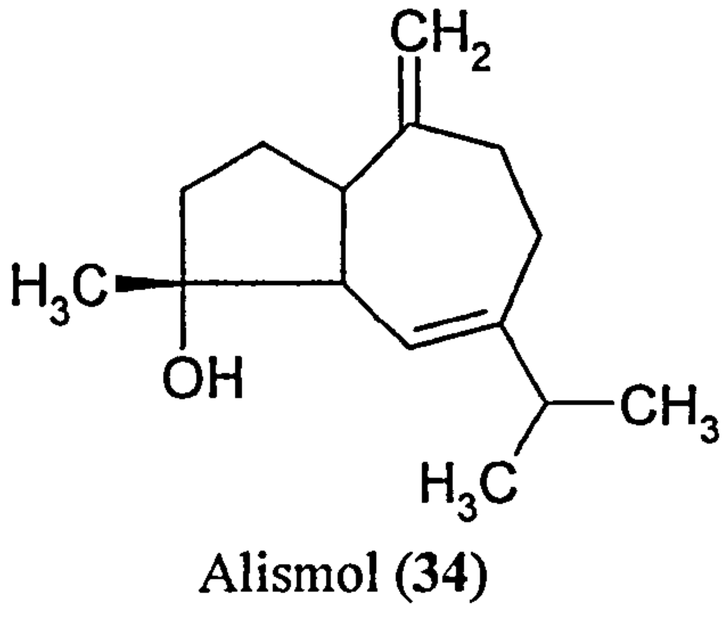
Alisol A (**28**): R=H
Alisol A monoacetate (**29**): R=OC-CH_3



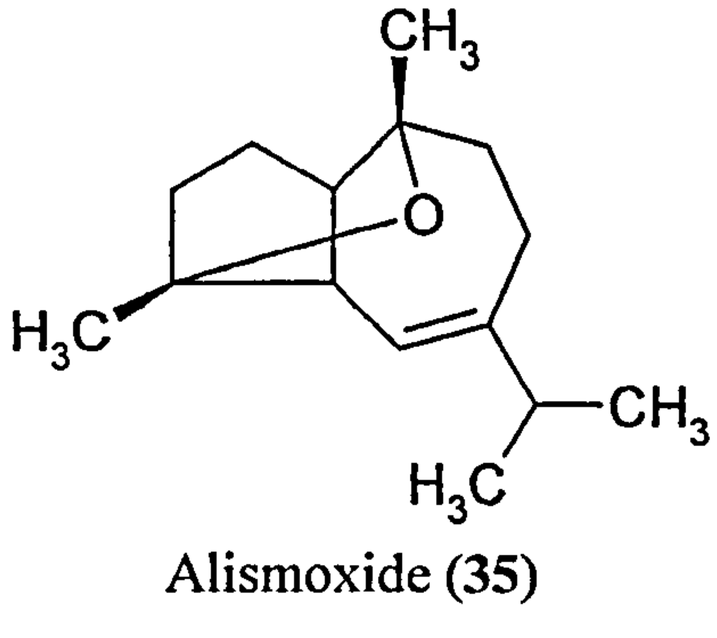
Alisol B (**30**): R=H
Alisol B monoacetate (**31**): R=OC-CH_3



Alisol C (**32**): R=H
Alisol C monoacetate (**33**): R=OC-CH_3



Alismol (**34**)



Alismoxide (**35**)

Toki-shakuyaku-san (TSS) is a prescription used in traditional Japanese medicine (TJM), which consists of six herbs, including *A. orientalis* root. TSS is reported to improve learning and memory deficits *in vivo*, to stimulate ChAT activity in the cortex, and to stimulate nicotinic receptor synthesis in brain (Itoh *et al.*, 1996b). Experiments have shown that it ameliorated the scopolamine-induced deficit in ACh (1) levels in mouse brain, but did not influence ACh (1) levels in untreated mice (Itoh *et al.*, 1996b). These findings suggest that TSS may promote favourable cognitive effects in AD, as cholinergic function is impaired. The effects observed with TSS may be due to one or more of the herb components and further investigations are required to establish the influence of *A. orientalis* root alone on memory disorders and cholinergic function.

Triterpenes (e.g. alismaketones B 23-acetate and C 23-acetate) from *A. orientalis* root, inhibited NO production in macrophages (Matsuda *et al.*, 1999) and it is noteworthy that inhibition of NO production by microglia in the CNS may provide neuroprotection in AD.

A. orientalis root and constituents are also reported inhibit platelet aggregation, to have immunological, hypoglycaemic and hypotensive activities, and to treat hypercholesterolaemia (Shimizu *et al.*, 1994; Tomoda *et al.*, 1994; Tang and Eisenbrand, 1992). Activity against hypercholesterolaemia may lower the risk of cardiovascular disease, and perhaps protect against ischaemia-induced dementia.

1.4.2.2 *Apocynum lancifolium* L. (Apocynaceae)

Apocynum lancifolium leaf and root are used in TCM for hypotensive, cardiotonic and diuretic effects. The root and leaf are recommended for hypertension, heart disease, neurosis, oedema and nephritis, and are also used for chronic bronchitis and influenza (Chang and But, 1987; Huang, 1993). In the northern provinces of China, the tea is used to ease dizziness (Jianming, 1988).

The root contains cardiac glycosides (e.g. cymarin (**48**), strophanthidin (**47**), K-strophanthin-β (**49**)) and hydroxyacetophenone; the stem and leaves also contain cardiac glycosides, flavones (e.g. quercetin (**104**)), flavonol-*O*-glycosides (e.g. hyperin), phenylpropanoid substituted flavan-3-ols (e.g. apocynins A - D), lauric acid, phenolic compounds, steroid saponins, coumarins (e.g. scopoletin) and triterpenes

(e.g. α-amyrin and lupeol) (Chang and But, 1987; Duke and Ayensu, 1995; Fan *et al.*, 1999; Huang, 1993; Jianming, 1988; Miaohua and Fengshan, 1991).

The majority of research on *A. lancifolium* has focused on the cardioactive properties of the herb and its cardiac glycosides. The leaves are reported to slow heart rate and cardiac contractility, but high doses (1g/kg administered to cats and dogs) have been associated with arrhythmias, and administration of a 6% solution of the cardiac glycosides caused ventricular tachycardia and death (Chang and But, 1987). Administration of the root decoction to cats and administration of the cardiac glycosides to dogs, increased coronary blood flow and myocardial O_2 consumption (Chang and But, 1987). The latter vasodilatory effects indicate that cerebral blood flow may also be enhanced, although cardiotoxicity may restrict clinical use. A tea prepared from the leaves was administered to 196 and 427 patients with hypertension and a reduction in blood pressure was observed in 82% and 89% of patients respectively (Chang and But, 1987; Jianming, 1988).

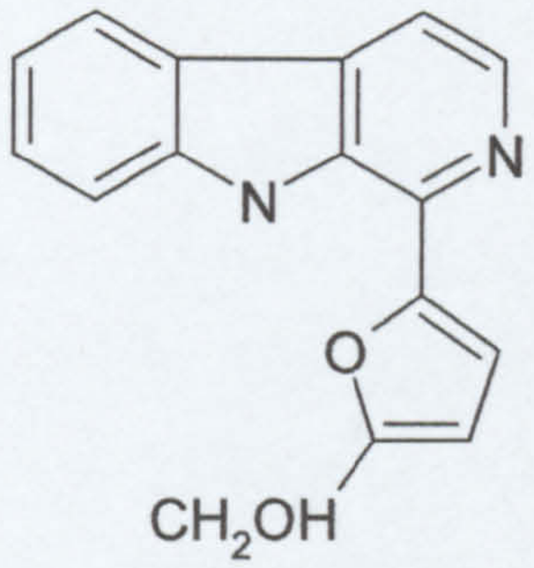
There is a comparative lack of studies regarding the potential memory-enhancing and anti-aging effects of *A. lancifolium*. The leaf extract increased SOD activity and prevented damage to DNA (Hao *et al.*, 1988; Ma and Chen, 1989), which suggests the leaf may have anti-oxidant activity.

The leaf is also reported to be effective in the treatment of chronic bronchitis and viral infections, hyperlipidaemia and is also sedative (Chang and But, 1987; Chen and Liu, 1991; Jianming, 1988; Ma and Chen, 1989; Miaohua and Fengshan, 1991).

1.4.2.3 *Codonopsis pilulosa* (Franch) Nannf. (Campanulaceae)

In TCM, *Codonopsis pilulosa* root is used for various disorders, including amnesia, anorexia, asthma, cancer and insomnia, and is believed to promote blood circulation and enhance vitality (Duke and Ayensu, 1995).

The water-soluble components present in the root include amino acids (e.g. aspartic acid, proline and valine) and sugars (e.g. fructose and inulin) (Jiang *et al.*, 1986; Liu and Wang, 1983; Sha *et al.*, 1988). Other compounds identified in the root include saponins, alkaloids (e.g. perlolyrine (**36**), sterols (e.g. α-spinosterol), triterpenes and also mucilage, resin and an essential oil containing methyl palmitate, octadecane, nonadecane and heptadecane (Chang and But, 1987; Liao and Lu, 1987; Liu *et al.*, 1988; Tang and Eisenbrand).



Perlolyrine (**36**)

Figure 1.5. *Codonopsis pilulosa* plant.

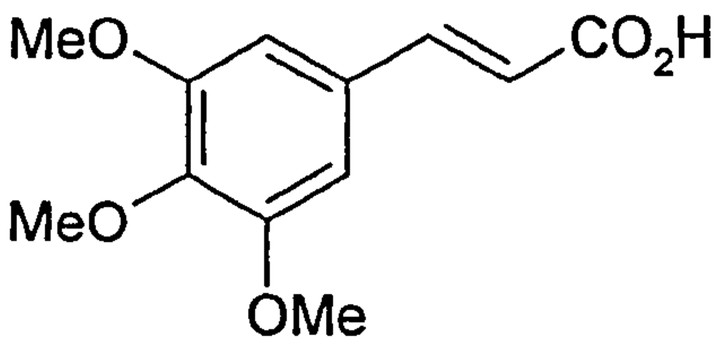
Pharmacological studies on *C. pilulosa* root to date have not included investigations regarding the reputed anti-amnesic effects. The root is reported to enhance reticuloendothelial phagocytosis and increase erythrocyte levels *in vivo*, to induce hyperglycaemia (due to the sugar content of the root) and hypotension *in vivo* and in clinical studies, the root is reported to treat neurosis (in combination with vitamin B), nephritis and gastric ulcers, and was also an anti-emetic (Chang and But, 1987).

1.4.2.4 *Polygala tenuifolia* Willd. (Polygalaceae)

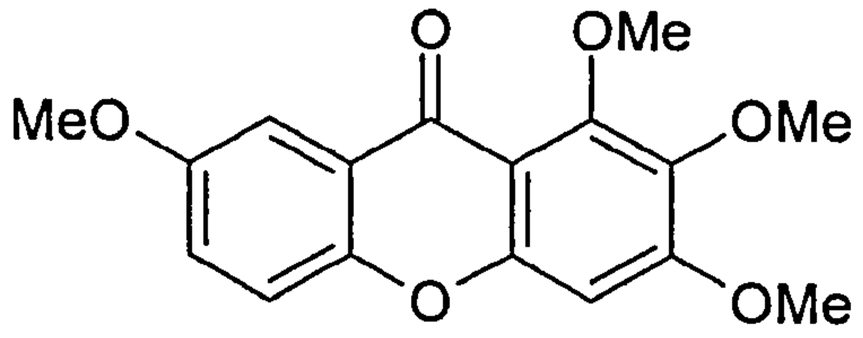
Polygala tenuifolia root is used in TCM as a cardiotonic and cerebrotonic, as an expectorant, sedative and tranquilliser, and for amnesia, forgetfulness, neuritis, nightmares and insomnia (Chang and But, 1987; Duke and Ayensu, 1995). According to the Chinese Materia Medica, the root is supposed to have special effect upon the will and mental powers, giving strength of character, improving understanding, strengthening the memory, and increasing physical powers (Chang and But, 1987; Duke and Ayensu, 1995).

Figure 1.6. *Polygala tenuifolia* root used in the present study.

Constituents isolated from the root of this herb include the alkaloid tenuidine, the phenylpropanoid 3, 4, 5-trimethoxy-cinnamic acid (**37**), triterpene saponins (including prosapogenin, onjisaponins A - G and tenuifolin (**67**)), phenolic glycosides (tenuifolisides A - F), oligosaccharides (tenuifolioses A - P), xanthone derivatives (e.g. 1, 2, 3, 7-tetramethoxyxanthone (**38**)), fixed oil and resin (Chang and But, 1986; Fujita *et al.*, 1992; Huang, 1993; Ikeya *et al.*, 1991a; Ikeya *et al.*, 1991b; Ikeya *et al.*, 1994; Miyase *et al.*, 1991; Miyase *et al.*, 1992; Sakuma and Shoji, 1981a; Sakuma and Shoji, 1981b; Tang and Eisenbrand, 1992; Trease and Evans, 1996).



3, 4, 5-Trimethoxy-cinnamic acid (**37**)

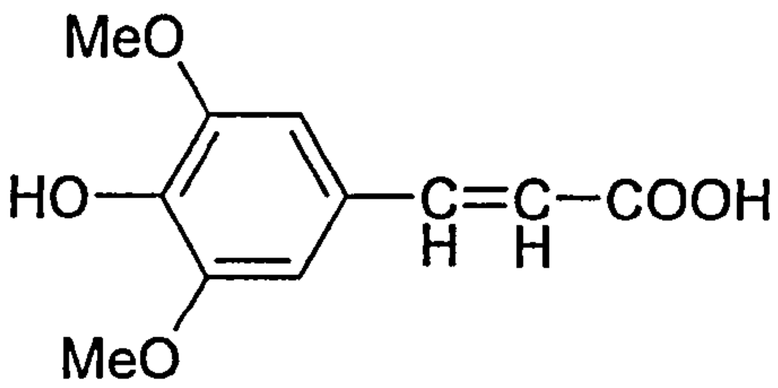


1, 2, 3, 7-Tetramethoxyxanthone (**38**)

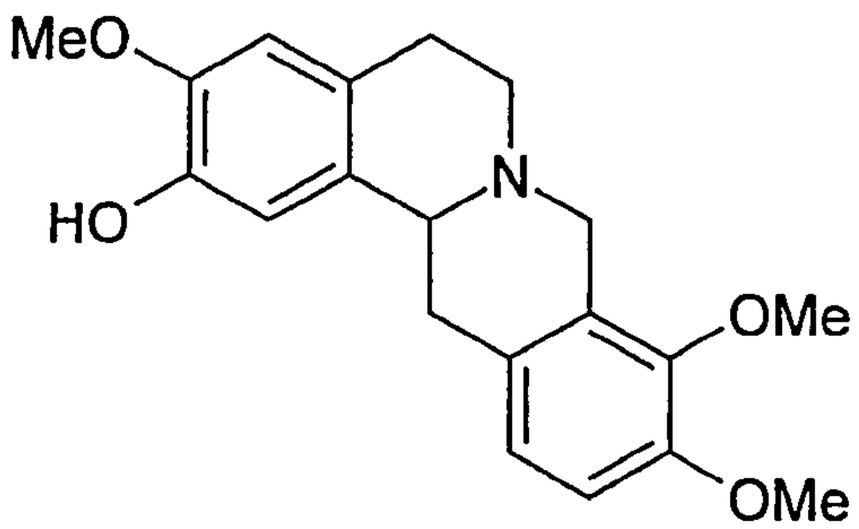
There have been numerous studies regarding the reputed memory enhancing potential of *P. tenuifolia* root. For example, the traditional Chinese prescription DX-9386, composed of four herbs, including *P. tenuifolia* root, has shown favourable effects in relation to AD symptoms in several animal models. DX-9386 improved motor activity, reduced lipid peroxidation, ameliorated memory impairment and prolonged the lifespan of senescence accelerated mice and, ameliorated the ethanol- and scopolamine-induced memory impairment in mice (Nishiyama *et al.*, 1994a; Nishiyama *et al.*, 1994b; Nishiyama *et al.*, 1994c; Zhang *et al.*, 1994b). Further investigations are required to clarify the contribution of each of the four herbs in DX-9386 to the observed pharmacological activities.

Kami-utan-to (KUT), a prescription containing thirteen herbs including *P. tenuifolia* root and also *Z. jujuba* fruit and seeds, is used in TJM to treat psychoneurological diseases. KUT dose-dependently upregulated ChAT activity and increased NGF secretion *in vitro*, improved passive avoidance behaviour and induced ChAT activity in the cerebral cortex of aged rats and in scopolamine-induced memory impaired rats *in vivo* (Yabe *et al.*, 1997; Yamada and Yabe, 1997). The effects on ChAT activity and NGF secretion *in vitro* were not as pronounced when treated with KUT in the absence of *P. tenuifolia* root, but *P. tenuifolia* root extract alone did upregulate ChAT activity and increase NGF secretion *in vitro* (Yabe *et al.*, 1997; Yamada and Yabe, 1997). The cinnamic acid derivative sinapinic acid (**39**), from *P. tenuifolia* root increased ChAT activity in the frontal cortex in brain-lesioned rats (Yabe *et al.*, 1997). These results indicate that *P. tenuifolia* root, particularly the cinnamic acid derivatives, significantly contributes to the pharmacological activities of KUT, and may explain the reputed beneficial effects of KUT in TJM. KUT treatment in AD patients is also reported to improve memory related behaviour (Yamada and Yabe, 1997).

A dopamine receptor ligand tetrahydrocolumbamine (**40**) has also been isolated from *P. tenuifolia* root (Shen *et al.*, 1994), but its potential agonistic or antagonistic activity *in vivo*, and any relevance to CNS disorders such as AD or Parkinson's disease, require further investigation.



Sinapinic acid (**39**)



Tetrahydrocolumbamine (**40**)

An aqueous extract of *P. tenuifolia* root inhibited IL-1 mediated TNF secretion by astrocytes *in vitro* (Kim *et al.*, 1998), which suggests the reputed favourable effects of the herb in CNS disorders, may also involve anti-inflammatory activity but, this requires confirmation *in vivo*. The aqueous extract also dose-dependently inhibited ethanol-induced IL-1 secretion *in vitro* (Koo *et al.*, 2000).

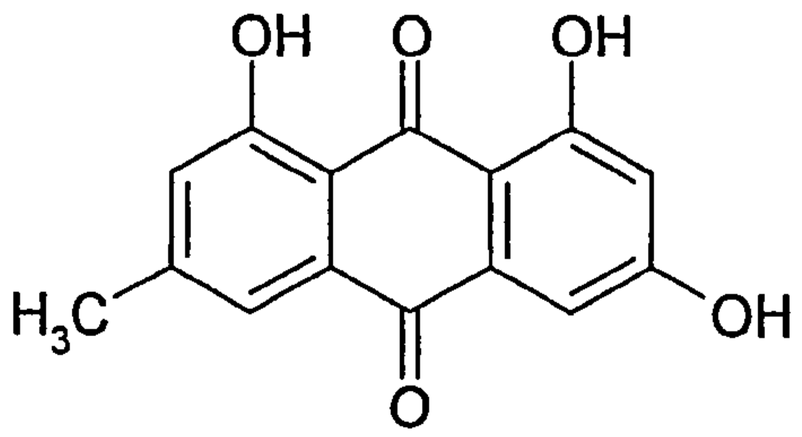
P. tenuifolia root is also reported to stimulate uterine contraction, to prolong hexobarbital sleeping time in mice (due to onjisaponon F) and, have anti-convulsant, anti-bacterial, hypotensive and expectorant properties (Chang and But, 1987; Huang, 1993; Tang and Eisenbrand, 1992).

1.4.2.5 *Polygonum multiflorum* Thunb. (Polygonaceae)

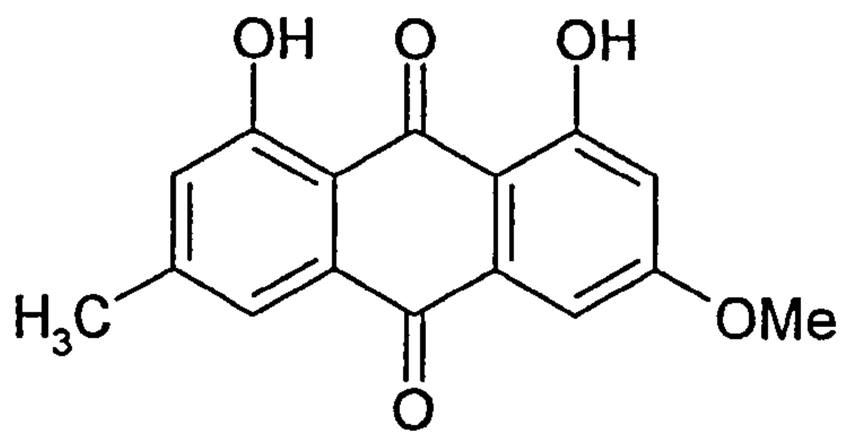
Polygonum multiflorum root is used in TCM for various conditions including constipation, dizziness, tinnitus, insomnia, neurosis, neurasthenia and in combination with three other herbs, forms one of China's great herbal tonics to maintain youthfulness and general good health; in the Chinese Materia Medica, the root is described as having wonderful restorative and reviving powers (Chang and But, 1986; Duke and Ayensu, 1995; Huang, 1993).

Compounds identified in *P. multiflorum* root include anthraquinones (e.g. emodin (**41**) and physcion (**42**)), stilbenes (e.g. resveratrol (**43**)), hydroxylated stilbene glycosides (e.g. 2, 3, 5, 4-tetrahydroxystilbene 2-*O*-β-D-glucopyranoside (**44**)) and

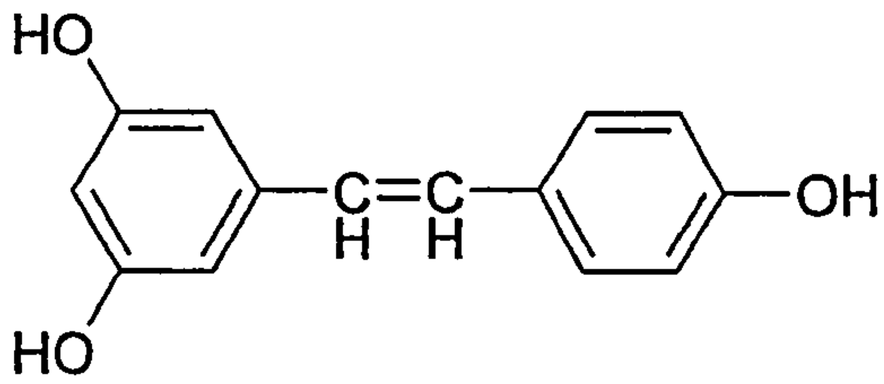
lecithin (Chang and But, 1986; Grech *et al.*, 1994; Harborne and Baxter, 1993; Huang, 1993; Nonaka *et al.*, 1982; Tang and Eisenbrand, 1992).



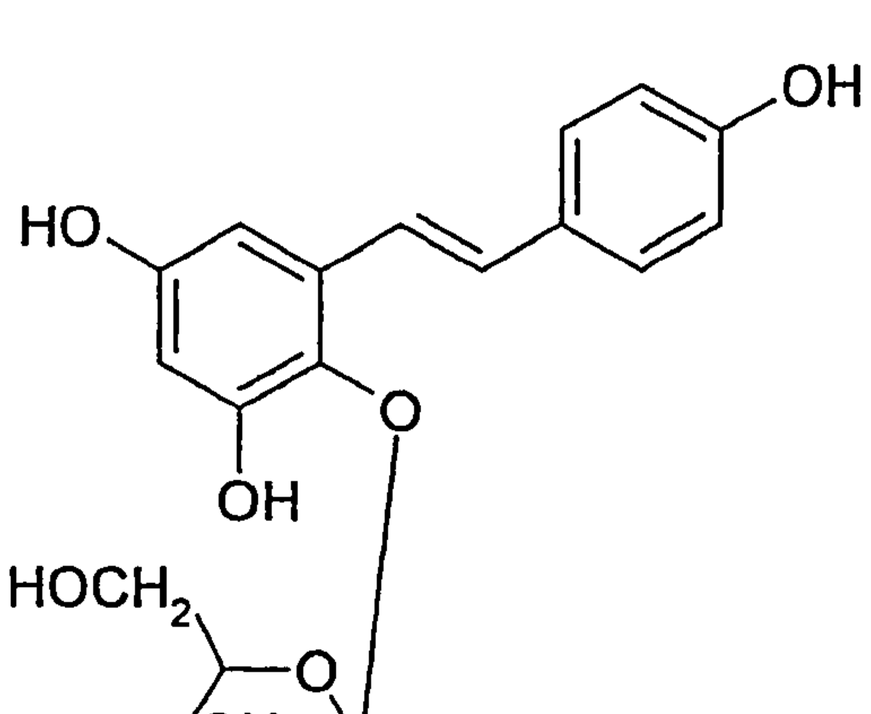
Emodin (**41**)



Physcion (**42**)



Resveratrol (**43**)



2, 3, 5, 4-Tetrahydroxystilbene 2-*O*-β-D-glucopyranoside (**44**)

Research conducted on *P. multiflorum* root has not focused on potential cognitive enhancing effects. The traditional Korean prescription Shao-yin-ren shi-quang-da-bu-tang (SDT), composed of ten herbs, including *P. multiflorum* root, restored the decline in cerebral choline content following oral treatment in senescence accelerated mice (Ma *et al.*, 1999). This result suggests that SDT may influence cholinergic function, which may be relevant in AD treatment. It is unknown which of the plants contribute

to this effect, or if compounds acted synergistically. One study showed that *P. multiflorum* root did not improve scopolamine-induced learning and memory deficits in rats (Hsieh *et al*., 2000), which suggests that potential cognitive enhancing effects of the root may not be related to cholinergic function. Other pharmacological activities of *P. multiflorum* root in relation to memory require further investigation.

Figure 1.7. *Polygonum multiflorum* plant.

Several studies have shown anti-oxidant and anti-inflammatory effects with *P. multiflorum* root and some of its isolated constituents, which may explain the anti-aging claim in TCM. Previous studies have shown *P. multiflorum* root (aqueous extract) to inhibit lipid peroxidation and scavenge free radicals (Hong *et al*., 1994; Ip *et al*., 1997). This suggests that the compounds responsible for the reported anti-oxidant effects are more polar than those compounds present in the less active ethanol extracts. A traditional Chinese prescription composed of *Angelica sinensis* and *Astragalus membranaceus* protected against rat myocardial ischaemia-reperfusion injury, which involves reactive oxygen species; this effect was enhanced by additional treatment with *P. multiflorum* root, but *P. multiflorum* root alone did not significantly protect against ischaemia-reperfusion injury (Yim *et al*., 2000). This study suggests *P. multiflorum* root may act synergistically with other phytochemicals to enhance anti-oxidant activity.

Resveratrol (**43**) and 2, 3, 5, 4'-tetrahydroxystilbene 2-*O*-β-D-glucopyranoside (**44**) are inhibitors of lipid peroxidation in rat liver microsomes (Kimura *et al*., 1983). These are phenolic compounds, a phenolic component being a common feature of

many plant derived anti-oxidants. The presence of the more polar glycosides would be expected in the aqueous extract of this plant so these compounds may explain the reported anti-oxidant activity of the aqueous extracts. A standardised extract from a related species *Polygonum cuspidatum*, consisting of 20% resveratrol (**43**) and 10% emodin (**41**) (Protykin), and the ethyl acetate fraction of an ethanolic extract from *P. multiflorum* is reported to have potent free-radical scavenging activity (Chen *et al.*, 1999; Sato *et al.*, 2000). Several other compounds isolated from *P. multiflorum* root are also known anti-oxidants; these include gallic acid, (+)-catechin, (+)-epicatechin, 3-*O*-galloyl-(-)-catechin and 3-*O*-galloyl-(-)-epicatechin (Bouchet *et al.*, 1998; Nonaka *et al.*, 1982).

P. multiflorum has been shown to inhibit acute ear oedema in mice (Cuéllar *et al.*, 1998), indicating anti-inflammatory activity. Another member of the Polygonaceae, *Polygonum cuspidatum*, has shown anti-inflammatory effects but the mechanism of action is unconfirmed. However resveratrol (**43**), present in the root extract of *P. cuspidatum*, suppresses NF-κB activation, which is a nuclear transcription factor that regulates the expression of genes involved in inflammation (Manna *et al.*, 2000). It is possible that *P. multiflorum* root may influence inflammatory mechanisms as resveratrol (**43**) has been identified in the root.

The anthraquinone emodin (**41**) is a potent inhibitor of NF-κB activation (Kumar *et al.*, 1998a) but shows no effect on COX-2 mRNA, and does not inhibit PGE_2 synthesis (Chen *et al.*, 2000). Stilbenes (resveratrol (**43**) and its glucoside, piceid) isolated from *Polygonum* spp. are reported to interfere with 5-hydroxy-6, 8, 11, 14-eicosatetraenoic acid (5-HETE), 17-hydroxyheptadecatrienoic acid (HHT) and thromboxane B_2 (TXB_2) synthesis in rat peritoneal leukocytes, attributed to the inhibition of COX and 5-LOX (Kimura *et al.*, 1985a). .

P. multiflorum root is reported to lower cholesterol, increase coronary circulation and antagonise tachycardia *in vivo*, and to have anti-bacterial properties; the root has also shown favourable effects in patients with neurasthenia, insomnia, hypercholesterolaemia or schizophrenia (Chang and But, 1986; Huang, 1993).

1.4.2.6 *Salvia miltiorrhiza* Bung. (Labiatae)

Throughout history the Labiate *Salvia miltiorrhiza* has been used for the treatment of a variety of medical conditions. The dried root of *S. miltiorrhiza* is red in colour, and

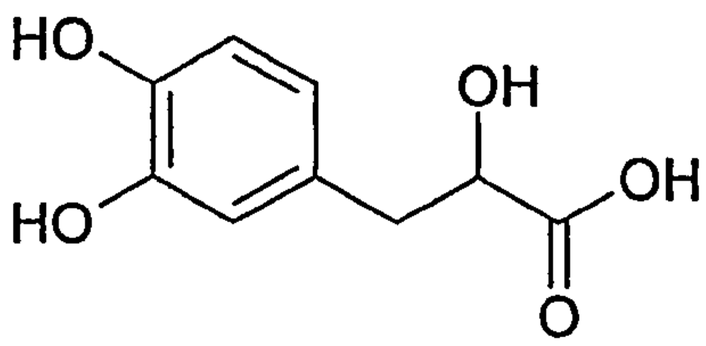
was therefore used in folk medicine for the management of blood disorders. It is prescribed in TCM to stabilise the heart and calm nerves (Huang, 1993). Official indications for the root include treatment of menstrual disorders, menostasis, menorrhagia, blood circulation disorders, angina pectoris, insomnia, neurasthenia and alleviation of inflammation (Tang and Eisenbrand, 1992).

Figure 1.8. *Salvia miltiorrhiza* plant.

Figure 1.9. *Salvia miltiorrhiza* root used in the present study.

Constituents identified in *S. miltiorrhiza* root include vitamin E and numerous diterpenoids (e.g. tanshinones I (**115**) and IIa (**116**), cryptotanshinone (**117**),

tanshindiols (A (**119**) and B (**120**)), isotanshinones (I (**121**) and II (**122**)), neocryptotanshinone II (**118**)) (Huang, 1993; Ikeshiro *et al.*, 1991; Kusami *et al.*, 1985; Lin and Chang, 2000; Luo *et al.*, 1986; Tang and Eisenbrand, 1992). Several water-soluble compounds have been isolated from the aqueous extract of *S. miltiorrhiza* root, including danshensu (**45**) (Chen and Liu, 1980), the salvianolic acids A (**123**) (Li *et al.*, 1984), B (**124**) and C (**125**) (Ai and Li, 1988) and rosmarinic acid (RA) (**108**) (Huang *et al.*, 1992; Huang, 1993).



Danshensu (**45**)

S. miltiorrhiza, or Chinese sage, has been the subject of thorough investigation, and consequently numerous pharmacological activities that may be relevant in CNS disorders, including AD, have been identified. *S. miltiorrhiza* has been employed for the treatment of cerebral vascular disease, and there are several studies to investigate possible mechanisms for the protective effect of *S. miltiorrhiza* against cerebral ischaemia.

S. miltiorrhiza root has been implicated in attenuating dysfunction of vasoactive intestinal peptide (VIP) (Kuang *et al.*, 1989), a neuropeptide distributed within the gastro-intestinal tract and central nervous system (CNS). VIP may participate in the changes that occur in cerebral ischaemia. Distribution abnormalities of the neuropeptide substance P have been associated with some CNS disorders, including AD. Decreased levels of substance P have been suggested as a consequence of neuron damage following cerebral ischaemia; *S. miltiorrhiza* root has been implicated in protecting neurons from ischaemia (Kuang *et al.*, 1991), and so may actively protect against cerebral ischaemia and perhaps other CNS disorders via this mechanism.

Other beneficial effects of *S. miltiorrhiza* root against cerebral ischaemia have also been explored. *S. miltiorrhiza* root may inhibit neuronal cell death by inhibition of presynaptic glutamate release (Kuang and Xiang, 1994), and it has been suggested that inhibition of NO formation may also explain CNS protective effects observed

with *S. miltiorrhiza* root (Kuang *et al.*, 1996a). It should also be considered that the biological function of NO has been suggested to involve the effects of excitatory amino acids, including their effects on brain development, learning and memory (Moncada *et al.*, 1991). This physiological role may aid the explanation of the effects of *S. miltiorrhiza* root on the CNS. Further investigations indicate *S. miltiorrhiza* root may modify ischaemic cell changes by modulating somatostatin, a CNS neuropeptide that has implicated in learning and memory (Kuang *et al.*, 1993).

S. miltiorrhiza root may offer an additional therapeutic approach to management of stroke and ischaemia. Reperfusion to aid recovery of ischaemia can cause further brain damage. During reperfusion, metabolism of free fatty acids from the breakdown of lipid membranes during ischaemia has been proposed to generate oxygen free radicals, leading to further brain injury (Traystman *et al.*, 1991). *S. miltiorrhiza* root has been shown to offer protection against this process by reducing lipid peroxidation (Kuang *et al.*, 1996b; Liu *et al.*, 1992; Peigen *et al.*, 1996; Zhao *et al.*, 1996).

The anti-oxidant effects of *S. miltiorrhiza* root have been studied and several compounds have been identified with significant anti-oxidant activity. Several quinones isolated from *S. miltiorrhiza* root have demonstrated an anti-oxidant effect in lard, with dihydrotanshinone, tanshinone I (**115**), methylene tanshinquinone and cryptotanshinone (**117**) providing significant antioxidant activity; tanshinone IIa (**116**) has shown no anti-oxidant activity (Weng and Gordon, 1992; Zhang *et al.*, 1990). Other components of *S. miltiorrhiza* root have displayed anti-oxidant effects including salvianolic acid A (**123**) (a compound found to protect against memory impairment induced by cerebral ischaemia reperfusion in mice (Guanhua and Juntian, 1997)), salvianolic acid B (**124**) and rosmariquinone (also known as miltirone; it has been isolated from another labiate, *Rosmarinus officinalis*) and several other phenolic compounds (Huang and Zhang, 1992; Kang *et al.*, 1997; Liu *et al.*, 1992; Weng *et al.*, 1992). The compounds that show anti-oxidant effects may be also useful in AD therapy.

'*Salvia compositus*' is a herbal mixture of the Chinese herbs *S. miltiorrhiza* and *Delbergia odorifera*, traditionally used for management of coronary heart disease (Fan *et al.*, 1979). Investigations suggest this herbal remedy has a potential role in anti-oxidation of lipids (Zhang *et al.*, 1994a), and in amelioration of cerebral oedema (Kuang *et al.*, 1995), providing further evidence for therapeutic advantage in cerebral disorders.

'*Salvia compositus*' has shown effects on electrical activities of the cerebral cortex, showing a CNS depressant action (Fan *et al.*, 1979). There are reports of *S. miltiorrhiza* root being analgesic and sedative. A reduction in the spontaneous activity of mice, and increased duration of the hypnotic action induced by chloral hydrate and barbiturates in the presence of *S. miltiorrhiza* root have been observed (Huang, 1993; Chang and But, 1986). Further investigation has established a structure-activity relationship of rosmariquinone (miltirone), a quinone isolated from *S. miltiorrhiza* root, as an active central benzodiazepine receptor ligand (Chang *et al.*, 1991). Rosmariquinone and perhaps other quinones from *S. miltiorrhiza* may explain the tranquillising effects observed, and may aid the development of a novel class of anxiolytic agents.

Tanshinones isolated from *S. miltiorrhiza* root have demonstrated anti-inflammatory activity in mice and were active against 5-LOX in porcine leukocytes, but were not as active as the crude extracts (Chang and But, 1986; Paulus and Bauer, 2000). The tanshinones are also reported to show weak oestrogenic activity (Chang and But, 1986). These activities require further investigation for confirmation, but may be relevant in AD therapy.

Other activities reported to occur with *S. miltiorrhiza* root include anti-bacterial activity, inhibition of platelet aggregation, potential as an anti-ulcer treatment, and efficacy in angina and hypertensive patients, and in the treatment of hepatitis, infectious diseases and tonsillitis (Fang *et al.*, 1976; Huang, 1993; Murakami *et al.*, 1990; Onitsuka *et al.*, 1983; Tang and Eisenbrand, 1992).

1.4.3 Traditional European Medicinal Plants

1.4.3.1 *Convallaria majalis* L. (Liliaceae)

Convallaria majalis has been used in traditional European medicine as a diuretic and cardiotonic, and was also used as an embrocation for sprains and rheumatism and the aqueous extract was used for inflammatory eye conditions (Grieve, 1984). Herbalists throughout history have suggested that *C. majalis* may be useful in cardiovascular and cognitive disorders.

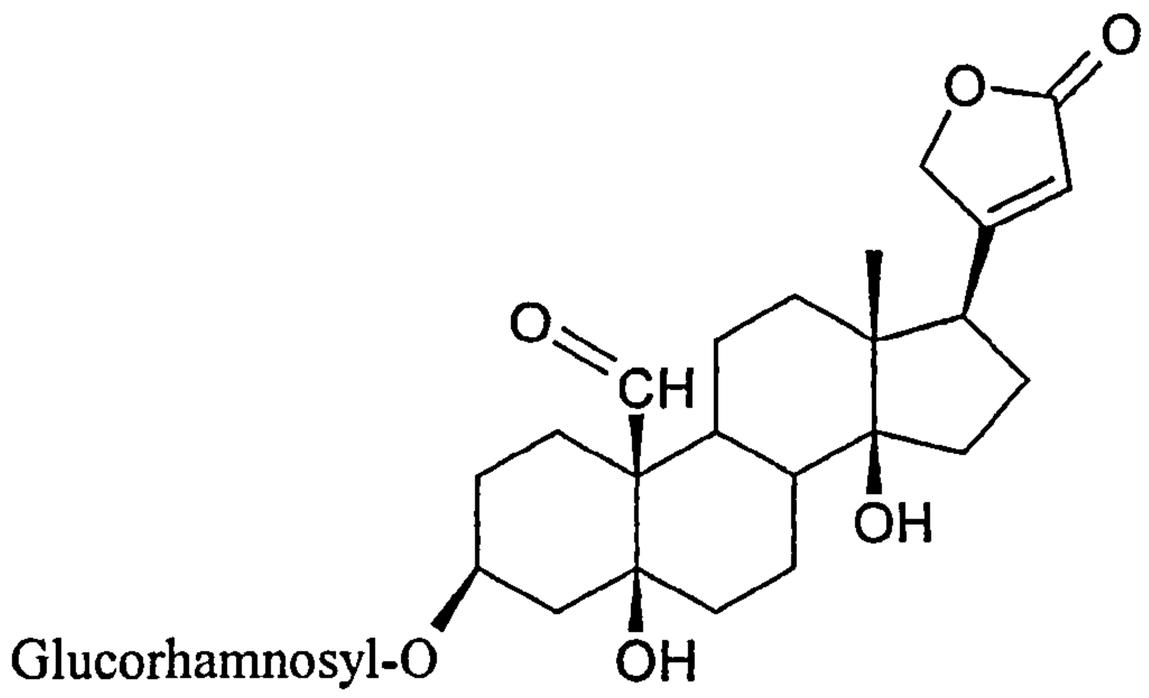
'It doth strengthen the Memorie and comforteth the Harte'

-Dodoens, 1560 (Grieve, 1984).

'It without doubt strengthens the brain and renovates a weak memory'

-Culpepper (Grieve, 1984).

C. majalis contains 0.2% - 0.3% of at least forty different types of cardiac glycoside, including convallamarin, convallatoxin (**50**), convalloside (**46**), sarhamnoloside and tholloside (Saxena and Chaturvedi, 1992; Jurenitsch *et al.*, 1982; Kopp and Kubelka, 1982; Schrutka-Rechtenstamm *et al.*, 1986). Flavonoids, steroidal saponins, tannins and volatile oil constituents are also reported to be present in the herb (Frohne and Pfänder, 1984; Grieve, 1984; Weiss, 1988). The phytochemical composition may vary, depending on the geographical source (e.g. in western and north-western European plants, the primary cardenolide present is convallatoxin (**50**), but in central European plants, convalloside (**46**) is often the main cardenolide present) (Frohne and Pfänder, 1984).



Convalloside (**46**)

There has been considerable research regarding the cardioactive properties of *C. majalis* and its cardenolides (David *et al.*, 1982; Gupta and Chopra, 1987); activity that was indicated by the herbalists Culpepper and Dodoens. In some European countries the cardiac glycosides are used as an alternative to *Digitalis* spp. for cardiovascular disorders such as heart failure (Dewick, 1997) but all parts of *C. majalis* are toxic, which limits clinical use. Toxicity, which may be fatal, occurs due

to the arrhythmogenic cardenolides (Cooper and Johnson, 1984; David *et al*., 1982). Convallatoxin (**50**) has low oral bioavailability (Bruneton, 1995), which may reduce toxic effects but could also limit efficacy.

In contrast there is a comparative lack of pharmacological studies to investigate the reputed cognitive enhancing effects of *C. majalis*. Further research is necessary to identify any pharmacological basis for the reputed activities of *C. majalis*, other than its cardioactive effects.

1.4.3.2 *Melissa officinalis* L. (Labiatae)

Melissa officinalis leaf has been used as a medicinal plant for more than 2000 years. In the Middle Ages, it was used to soothe tension, dress wounds and to cure toothache (McVicar, 1994). In traditional European medicine *M. officinalis* was used as a calming and strengthening remedy and to treat digestive disorders, migraines, melancholia, neuroses and hysteria and the plant has been acclaimed for promoting long life and for restoring memory (Bisset, 1994; Kenner and Requena, 1996; McVicar, 1994; Yarnell, 1998). In Arabic medicine it was used to treat depression (McVicar, 1994). The Commission E Monograph in Germany approves the use of *M. officinalis* for nervous insomnia and gastro-intestinal disorders. In modern alternative medicine, *M. officinalis* essential oil is used in aromatherapy to alleviate depression and insomnia (McVicar, 1994).

Figure 1.10. *Melissa officinalis* plant.

***Melissa officinalis*: 'Good for disorders of the head and stomach'**

- John Hill, 1751 (Crellin and Philpott, 1990).

Approximately 250 constituents are reported to have been isolated from *M. officinalis*, with almost 200 of these being essential oil constituents, chiefly monoterpenes (>60% of the essential oil composition) and sesquiterpenes (>35% of the essential oil composition) (Bisset, 1994; Schultze *et al.*, 1993). The essential oil from *M. officinalis* is localised in glandular trichomes in the leaves, as with other Labiates. The primary monoterpenes identified in the essential oil include citronellal (**68**), geranial (**70**) and neral (**71**), and the main sesquiterpenes identified include caryophyllene (**82**), cubebene (**80**) and germacrene-D (Bisset, 1994; Guenther, 1949; Şarer and Kökdil, 1991; Tittel *et al.*, 1982). Tannins (e.g. caffeic acid (**106**) and related derivatives), triterpenes, phenylpropanoids (e.g. chlorogenic acid (**107**) and rosmarinic acid (RA) (**108**)) and flavonoids (e.g. apigenin and luteolin (**114**)) have also been identified in *M. officinalis* leaf (Agata *et al.*, 1993; Bisset, 1994; Bruneton, 1995; Heitz *et al.*, 2000; Mulkens and Kapetanidis, 1987; Schultze *et al.*, 1993; Yarnell, 1998).

M. officinalis has been the subject of research regarding its potential as a sedative and anxiolytic, activities that may be appropriate to provide symptomatic relief for behavioural problems such as agitation in AD. *M. officinalis* leaf was reported to alleviate mild anxiety and nervousness in a double-blind study, and in combination with *Valeriana officinalis* root, was reported to be as effective as triazolam, and did not cause drowsiness or impair concentration the next day (Yarnell, 1998). A hydroalcoholic (30% ethanol) extract of *M. officinalis* leaf was sedative in mice and potentiated barbiturate induced sleep, but the *M. officinalis* essential oil did not demonstrate these sedative effects (Soulimani *et al.*, 1991).

Other activities of *M. officinalis* leaf extracts that may be useful for AD therapy include anti-oxidant effects (Hohmann *et al.*, 1999; Lamaison *et al.*, 1991; Marinova and Yanishlieva, 1997; Tagashira and Ohtake, 1998) and binding to muscarinic and nicotinic receptors *in vitro* (Perry *et al.*, 1996; Wake *et al.*, 2000), which suggests that favourable effects on cholinergic function may occur in AD patients.

Other activities identified in *M. officinalis* essential oil include anti-bacterial, anti-fungal, anti-parasitic and anti-viral activities (e.g. activity against *Herpes simplex labialis*) and the potential for treatment of hyperthyroidism (Koytchev *et al.*, 1999;

Larrondo *et al*., 1995; Mikus *et al*., 2000; Schultze *et al*., 1993; Yarnell, 1998). The essential oil is also widely used in cosmetics and perfumery, for its citrus odour.

1.4.3.3 *Rosmarinus officinalis* L. (Labiatae)

Rosmarinus officinalis leaf was used in European folk medicine as an application for wounds and for eczema, and has also been used traditionally as a carminative, to stimulate appetite, for nervous depression and had a reputation for strengthening the memory and enhancing mental alertness (Bisset, 1994; Grieve, 1984; Kenner and Requena, 1996). The herb has also been used as an analgesic, anti-rheumatic, diuretic, expectorant and for treatment of respiratory disorders (Al-Sereiti *et al*., 1999).

Figure 1.11. *Rosmarinus officinalis* plant.

***Rosmarinus officinalis*: 'It helpeth the brain, strengtheneth the memorie, and is very medicinable for the head'**

Roger Hacket, 1607 (Grieve, 1984).

R. officinalis leaf constituents identified include flavonoids (e.g. luteolin (**114**), apigenin) and corresponding glycosides, phenolic compounds (e.g. caffeic acid (**106**)

and chlorogenic acid (**107**)), diterpenoid (e.g. carnosol (**142**)) and triterpenoid compounds (e.g. oleanic acids, α- and β-amyrin and rofficerone), and a volatile oil composed mainly of monoterpenes (Bisset, 1994; Ganeva *et al.*, 1993; Newall *et al.*, 1996; Okamura *et al.*, 1994). The major constituents of *R. officinalis* essential oil are 1, 8-cineole (**56**) (15-30%), camphor (**57**) (15-25%), α-pinene (**53**) (up to 25%) and other monoterpenes (e.g. borneol (**58**), bornyl acetate (**51**), limonene (**73**)) (Bisset, 1994; Panizzi *et al.*, 1993; Trease and Evans, 1996).

R. officinalis has been investigated for various biological activities, including some that are perhaps relevant in the treatment and prevention of AD, but studies that show direct effects of the herb on cognitive function are lacking. The essential oil stimulated respiratory and locomotor activity in mice and the oil constituent 1, 8-cineole (**56**) was detected in blood, following inhalation (Kovar *et al.*, 1987). The pharmacological basis and the oil constituents responsible for these observations remain unclear, but the results indicate that oil constituents may reach the systemic circulation following inhalation, and may therefore potentially cross the BBB and show pharmacological activity in the brain (lipid-soluble molecules with a molecular mass <400Da - 600Da may readily cross the BBB (Pardridge, 1998)). The essential oil is also reported to improve circulation (Al-Sereiti *et al.*, 1999). This suggests the oil may also improve cerebral blood flow and perhaps cognitive function, but this effect requires investigation. *R. officinalis* essential oil also weakly inhibited AChE *in vitro* (Perry *et al.*, 1996), but this activity requires confirmation *in vivo*.

R. officinalis leaf extracts and constituents (e.g. diterpenes including carnosic acid (**143**)) have demonstrated anti-oxidant activity (Al-Sereiti *et al.*, 1999; Haraguchi *et al.*, 1995; Joyeux *et al.*, 1995; Lamaison *et al.*, 1991; Masaki *et al.*, 1995). The pharmacological basis for the reputed memory-enhancing effects of *R. officinalis* may be due to effects on cerebral blood flow, anti-oxidant activity or other unknown mechanisms, but further investigations are required to establish this.

R. officinalis essential oil is also reported to have anti-bacterial, anti-fungal, muscle relaxant and hyperglycaemic activities (Al-Hader *et al.*, 1994; Aqel, 1991; Mangena and Muyima, 1999; Newall *et al.*, 1996; Panizzi *et al.*, 1993) and the plant extracts were hepatoprotective (Al-Sereiti *et al.*, 1999; Fahim *et al.*, 1999; Hoefler *et al.*, 1987).

CHAPTER 2

Phytochemical Investigations

Plant material was extracted using solvents of varying polarity for assessment of activities in the bioassay investigations. Investigations were conducted to separate the chemical constituents of the dried leaf of *Convallaria majalis* to obtain fractions for analysis in the *in vitro* studies. *C. majalis* leaf was subjected to various separation techniques, including droplet counter-current chromatography (DCCC), flash column chromatography (FCC) and preparative thin layer chromatography (TLC). The fractions obtained using these separation methods were tested for inhibition of acetylcholinesterase (AChE) activity *in vitro*, and were analysed using high performance liquid chromatography (HPLC), liquid chromatography-mass spectroscopy (LC-MS) and TLC, to obtain information regarding the phytochemistry of *C. majalis* leaf, and regarding the nature of the compounds responsible for anticholinesterase (antiChE) activity. Essential oils and oil constituents were analysed using gas chromatography-mass spectroscopy (GC-MS) to obtain information regarding their chemical composition and purity, prior to use in the bioassay investigations.

2.1 Phytochemical Methods

2.1.1 Materials

2.1.1.1 Materials: Herbs, Essential Oils and Commercially Obtained Constituents

Alisma orientalis root, *Codonopsis pilulosa* root, *Polygala tenuifolia* root, *Polygonum multiflorum* root, *Salvia miltiorrhiza* root (2 batches obtained) and *Ziziphus jujuba* fruit with seeds were supplied by the Chi Clinic, 10 Greycoat Place, London, SW1, England (herbs were imported from China). *Apocynum lancifolium* leaves and *Ziziphus jujuba* var. *spinosa* seeds were obtained from Mayway (UK) Ltd., 43 Waterside Trading Estate, Trumpers Way, Hanwell, W7 2QD, England (products

imported from 506 South Building, Zhong Guan Cun, Beijing, China); *Centella asiatica* leaves were obtained from Brome and Schimmer Ltd., 3 Greatbridge Road, Romsey, Hants, England; *Convallaria majalis* leaves were obtained from The Herbal Apothecary, High Street, Syston, Leicester, LE7 1GQ, England; *Melissa officinalis* dried leaf was obtained from G. Baldwin and Co., Walworth Road, London, SE17 1RW, England; *Rosmarinus officinalis* dried herb was obtained from Cotswold Health Products Ltd., Units 5 - 6 Tabernacle Road, Wotton-Under-Edge, Gloucestershire GL12 7EF, England; *Withania somnifera* root was obtained from PAK Chemicals Ltd., Karachi, India.

The essential oils were obtained as follows: *Rosmarinus officinalis* essential oil from G. Baldwin and Co., Walworth Road, London, SE17 1RW, England; *Melissa officinalis* essential oil from Fragrant Earth, Taunton, England; *Melissa officinalis* phytol extract (and crude extract), grown and extracted by Clwydian Fragrant Oils, Llyn-y-Pandy Lane, Pantymwyn, Clwyd, Wales.

The constituents citronellal (85-95%), DL-citronellol (95%), convallatoxin (80%), cymarin, γ-aminobutyric acid (GABA), geraniol (98%), α-humulene, (+)-limonene (97%) and nerol (98%) were purchased from Sigma, Fancy Road, Poole, Dorset, England; camphor (96%), caryophyllene oxide (90%), 1, 8-cineole (99%), citral (95%), eugenol (99%), (±)-linalool (97%), 6-methyl-5-hepten-2-one (99%), nerolidol (98%) and nonanal (95%) were purchased from Aldrich Chemical Company, New Road, Gillingham, Dorset, England; citral was also purchased from Lancaster (MTM Research Chemicals), Eastgate, White Lund, Morecambe, England; (+)-calarene (>99%), (+)-β-cedrene (97%), (-)-α-cubebene (≥ 98%), ocimene (97%) and (-)-*trans*-caryophyllene (99%) were purchased from Fluka Chemicals, The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT, England; hyperoside was obtained from Extra Synthèse, Fluorochem Ltd., Wesley Street, Old Glossop, Derbyshire, SK13 9RY, England. Chlorophyll (water soluble biological stain) was obtained from Hopkin and Williams, Essex, England.

2.1.1.2 Chromatography Materials

Normal phase silica gel TLC plates ($60F_{254}$) and silica gel ($60PF_{254}$) were obtained from Merck Ltd., Magna Park, Lutterworth, Leicestershire, England. Microgranular

cellulose powder (CC41) was obtained from Whatman International Ltd., Springfield Mill, Maidstone, England. Spray reagent chemicals were obtained as follows: anisaldehyde and chloramine T (*n*-chloro-*p*-toluene sulphonamide sodium salt) were obtained from Sigma, Fancy Road, Poole, Dorset, England; 3, 5-dinitrobenzene carboxylic acid, diphenyl-boric-amino-ethyl-ester complex (DPBAE) and bismuth subnitrate were obtained from Aldrich Chemical Company, New Road, Gillingham, Dorset, England; polyethylene glycol (PEG) 4000 and ninhydrin (idanetrione hydrate) were obtained from BDH Supplies, Poole, England; trichloroacetic acid was obtained from Fisher Scientific International Company, Loughborough, Leicestershire, LE11 5RG, England.

Biotage prepacked polyethylene cartridges (prepacked with KP-Sil™ 32-63µm, 60Å silica) and sample injection modules (SIM™) were obtained from Biotage UK Ltd., 15 Harforde Court, Foxholes Business Park, John Tate Road, Hertford, SG13 7NW, England. Solvents were obtained from BDH Supplies, Poole, England and Rathburn Chemicals Ltd., Hunter Boulevard, Magna Park, Lutterworth, Leicestershire, LE17 4XN, England.

2.1.2 Authentication of Plant Material

Apocynum lancifolium leaf and *Ziziphus jujuba* var. *spinosa* seeds were authenticated by Mayway (UK) Ltd., 43 Waterside Trading Estate, Trumpers Way, Hanwell, W7 2QD, England. *Centella asiatica* leaf was authenticated by Brome and Schimmer Ltd., 3 Greatbridge Road, Romsey, Hants, England. *Convallaria majalis* leaf was authenticated by The Herbal Apothecary, High Street, Syston, Leicester, LE7 1GQ, England. *Rosmarinus officinalis* dried herb was authenticated by Nicente Bravo, Spain. *Melissa officinalis* dried herb was authentictated by Viva Plantes, La Tourette, France. *Alisma orientalis* root, *Codonopsis pilulosa* root (voucher no. EBC 80745 36: 2/179), *Polygala tenuifolia* root, *S. miltiorrhiza* root (2 batches) (voucher no. EBC 80003 TCMK 4), *Ziziphus jujuba* fruit with seeds (voucher no. EBC 80090 TCMK 68) and *Ziziphus jujuba* var. *spinosa* seeds (voucher no. EBC 80646 TCMK 95) were authenticated (against plant material obtained from China by The Royal Botanic Gardens, Kew, England), and were confirmed to be the correct species, in collaboration with Ms Christine Leon and Professor Monique Simmonds, using morphological and microscopical analysis, at The Chinese Medicinal Plants

Authentication Centre, The Royal Botanic Gardens, Kew, Surrey, TW9 3AB, England. *Polygonum multiflorum* root was authenticated against *Gentiana scabra* root (voucher no. EBC 80728 TCMK 78) and *Rosmarinus officinalis* fresh herb was authenticated against an authentic sample of *Rosmarinus officinalis* fresh herb, in collaboration with Dr Renee Grayer at The Royal Botanic Gardens, Kew, Surrey, TW9 3AB, England, as described below.

2.1.2.1 Authentication of *Polygonum multiflorum* Root

0.5g *Polygonum multiflorum* root sample was cut, ground using a pestle and mortar and then added to 10ml CH_3COCH_3 in a sealed glass bottle. After 24hr the bottle contents were filtered and evaporated using a rotary evaporator. The residue was dissolved in 1ml MeOH and filtered through a 0.45µm filter (Gelman nylon acrodisc) prior to analysis using HPLC. HPLC parameters were as follows: column: C18 (Supelco Discovery), 250mm x 4.6mm (particle size: 5µm); pump: Waters 600E; UV detector: L-4000 (Merck Hitachi); wavelength: 210nm; solvent gradient: 75% A, 25% B (t=0min) then a linear gradient to 0% A, 100% B (t=20min), followed by 5min isocratic elution at 100% B then 75% A, 25% B (t=26min); where solvent A: 2% CH_3COOH in distilled H_2O; solvent B: MeOH : H_2O : CH_3COOH (18:1:1). This process was repeated for analysis of *Gentiana scabra* root. Refer to 2.2.1.1 for results and discussion.

2.1.2.2 Authentication of *Rosmarinus officinalis* Fresh Leaf

0.5g *Rosmarinus officinalis* leaf (sample: frozen leaf) was weighed and immersed in 15ml $(C_2H_5)_2O$ in a sealed glass bottle for external extraction of compounds of low polarity. 0.5g *R. officinalis* leaf (sample: frozen leaf) was weighed and immersed in 15ml 80% MeOH, then boiled for 5min for internal extraction of the more polar compounds, and the bottle was sealed. Both extracts were left for 24hr, prior to filtration. The filtered extracts were evaporated using a rotary evaporator. The residue was dissolved in 1ml MeOH and filtered through a 0.45µm filter (Gelman nylon acrodisc) prior to analysis using HPLC. HPLC parameters were as follows: column: C18 (Supelco Discovery), 250mm x 4.6mm (particle size: 5µm); pump: Waters 600E; UV detector: L-4000 (Merck Hitachi); wavelength: 210nm; solvent

gradient: 75% A, 25% B (t=0min) then a linear gradient to 0% A, 100% B (t=20min), followed by 5min isocratic elution at 100% B then 75% A, 25% B (t=26min); where solvent A: 2% CH_3COOH in distilled H_2O; solvent B: MeOH : H_2O : CH_3COOH (18:1:1). This process was repeated for analysis of the authentic *R. officinalis* plant material (fresh leaf).

The essential oil composition of the sample and authentic *R. officinalis* leaf was determined by thermal desorption-GC-MS, by placing two leaves in the desorption tube. Thermal desorption parameters were as follows: thermal desorber: Perkin Elmer ATD400; desorption temperature: 100°C; desorption time: 10min; focussing trap: Tenax TA80-100 mesh at 4°C; trap desorption: ballistic heating to 300°C; transfer line temperature: 200°C; outlet split enabled. GC parameters were as follows: GC: Perkin Elmer Autosystem; column: 30m x 0.22mm i.d., (particle size: 5µm) PE5-MS (Perkin Elmer); temperature program: 40°C - 260°C (4°C.min^{-1}); pressure: 15psi. MS parameters were as follows: Quadruple Perkin Elmer TurboMass; source: electron ionisation (70eV); temperature: 180°C; scan range: *m/z* 38 - 300. Refer to 2.2.1.2 for results and discussion.

2.1.2.3 Authentication of *Withania somnifera* Powdered Root

TLC analysis and authentication of *Withania somnifera* powdered root was conducted according to the method described by Upton, 2000. 1ml NH_3 (25% in H_2O) was added to 100mg powdered herb and the mixture was shaken. 10ml MeOH was added and the mixture was sonicated for 10s. The mixture was then heated to boiling in a water bath for 3min, and was then filtered. After evaporating to dryness using a rotary evaporator, the residue was reconstituted in 1ml MeOH.

10µl aliquots of this test solution were applied to a silica gel plate ($60F_{254}$) and developed using the mobile phase: $C_6H_5CH_3$: EtOAc : CHOOH (50:15:5). The plate was examined after spraying with 10% H_2SO_4 reagent in MeOH under UV light (at 254nm and at 366nm), and in daylight. Refer to 2.2.1.3 for results and discussion.

2.1.3 Extraction Methods of Plant Material

Plant material was ground using a grinding mill or kitchen blender and a known quantity (approximately 1g of plant material per 10ml of solvent) of plant material was extracted with solvents of different polarity as described below:

2.1.3.1 Hot Ethanolic Extraction

Plant material was refluxed in 96% EtOH for 20min.

2.1.3.2 Hot Aqueous Extraction

Plant material was refluxed in distilled H_2O for 20min.

2.1.3.3 Ethanolic Extraction at Room Temperature

Plant material was immersed in 96% EtOH in a glass vessel, sealed to prevent solvent evaporation, and stored at room temperature in the dark for one week.

2.1.3.4 Soxhlet Extraction

Soxhlet Extraction of Ziziphus jujuba Seeds

Sequential extraction of 4.3g of ground *Z. jujuba* seeds was performed using solvents of increasing polarity: 200ml PS, 200ml 96% EtOH and 200ml distilled H_2O for 5hr each time.

Soxhlet Extraction of Convallaria majalis Leaves

Sequential extraction of 25g of *C. majalis* leaves was performed using solvents of increasing polarity: 350ml C_6H_{14}, 350ml DCM and 350ml distilled H_2O for $2^1/_2$hr each time.

Each extract (2.1.3.1 - 2.1.3.4) was filtered and evaporated using a rotary evaporator (except aqueous extracts that were freeze dried). A known quantity of each residue was reconstituted with 96% EtOH to give extract concentrations ranging from 2mg/ml

to 20mg/ml, further dilution with 96% EtOH was performed to give a series of dilutions for use in the *in vitro* studies. Reconstituted extracts were stored at 4°C; dried extracts were stored at -20°C.

2.1.4 Methods for Preparation of Spray Reagents for TLC Analysis (Prepared According to Stahl, 1969)

Acidic Anisaldehyde: 0.5% anisaldehyde in MeOH : glacial CH_3COOH : concentrated H_2SO_4 (85:10:5). After spraying the plate was heated at 110°C for 5min - 10min and observed in daylight.

Chloramine T/Trichloroacetic Acid: Solution a: freshly prepared 3% chloramine T in distilled H_2O; solution b: 25% trichloroacetic acid in EtOH. 10ml solution a was added to 40ml solution b for use as the spray reagent. After spraying the plate was heated at 110°C for 7min and observed under UV light (366nm).

Dragendorff's Reagent: 100ml stock solution (consisting of 1.7g bismuth subnitrate, 20ml glacial CH_3COOH, 100ml 50% KI solution and 80ml distilled H_2O) and 200ml glacial CH_3COOH were made up to 1L with distilled H_2O. After spraying the plate was observed in daylight.

Kedde's Reagent: Kedde's solution a: 2% dinitrobenzoic acid in MeOH; Kedde's solution b: 3% KOH in MeOH. After spraying with solution a, then solution b, the plate was observed in daylight.

Natural Product Reagent (NPR): Solution a: 1% DPBAE in MeOH; solution b: 5% PEG 4000 in EtOH. After spraying with solution a, then with solution b, the plate was observed under UV light (366nm).

Ninhydrin: 0.2% ninhydrin in CH_3COCH_3. After spraying the plate was heated at 110°C for 5min - 10min and observed in daylight.

2.1.5 Method for the Preparation of Preparative TLC Plates

2.1.5.1 Preparation of Cellulose Plates

1mm thick cellulose plates were prepared by vigorously shaking approximately 40g cellulose powder with 100ml distilled H_2O for 4min - 5min. The resulting slurry was evenly spread across 5 glass plates and left overnight at room temperature to dry.

2.1.5.2 Preparation of Silica Gel Plates

1mm thick silica gel plates were prepared as follows: 90g silica gel (Keiselgel $60FF_{254}$) was dissolved in 200ml distilled H_2O and shaken vigorously for 4min - 5min. The resulting slurry was evenly spread across 5 glass plates and left overnight at room temperature to dry. Plates were placed in a TLC tank and MeOH was run to the top of each plate; the top 1.5cm - 2.0cm of silica on each plate was removed to eliminate contaminants. Plates were dried in an oven (60°C) for 45min to evaporate any remaining solvent.

2.1.6 Analysis of *Convallaria majalis* Leaf: Separation Methods

2.1.6.1 Flash Column Chromatography of *C. majalis* Leaf Extract (a)

An ethanolic extraction at room temperature of 6g *C. majalis* leaf yielded 0.609g of extract, which was reconstituted in 3ml MeOH, mixed with 4g silica gel ($60PF_{254}$), ground in a mortar and allowed to dry overnight. The silica coated sample was applied to a Biotage SIM™ (35ml volume). As solvents of increasing polarity (C_6H_{14} (100%); DCM (100%); DCM : CH_3COCH_3 (1:1); CH_3COCH_3 : MeOH (1:1) and MeOH (100%)) were added (500ml of each) under pressure (N_2: 5psi), compounds were passed through a Biotage prepacked column (i.d.: 15cm x 12mm, 8g); flow rate: $5ml.min^{-1}$. 55 fractions were collected and monitored using TLC and similar fractions were pooled to give 16 fractions (F1a - F16a). Each fraction was evaporated using a rotary evaporator, then final traces of solvent removed by blowing with N_2 at room temperature. Fractions were weighed and diluted in 96% EtOH to give a final

concentration of 10mg/ml. Fractions were stored at 4°C. Refer to 2.2.2.1 and 2.2.2.3 for results and discussion.

2.1.6.2 Flash Column Chromatography of *C. majalis* Leaf Extract (b)

5g of an ethanolic extract of *C. majalis* leaf (extracted at room temperature) was partitioned between 375ml DCM and 375ml H_2O. This process was repeated twice. The DCM fraction (1) was evaporated to dryness and stored at -20°C. The aqueous fraction was freeze dried, and further partitioned between 250ml EtOAc and 250ml H_2O (Figure 2.1). The EtOAc fraction (2) was evaporated to dryness, and the aqueous fraction (3) was freeze dried; extracts were stored at -20°C.

Fractions (1, 2, 3) were analysed using TLC (stationary phase: silica gel ($60F_{254}$); mobile phase: $C_6H_5CH_3$: EtOAc : MeOH (5:6:4); spray reagent: acidic anisaldehyde). Fraction 1 (the DCM layer) was also compared with the crude ethanolic extract of *C. majalis* leaf and with F8a (from FCC (a)) by application to silica gel ($60F_{254}$) plates. This was conducted to determine if the compounds present in the active F8a were also present in the DCM layer of the ethanolic extract. Compounds not of interest (i.e. those absent in the active fraction) could be eliminated, permitting a higher yield of active compounds in the FCC separation. Plates were developed using $C_6H_5CH_3$: EtOAc : MeOH (5:6:4) as the mobile phase, and examined using 2 different spray reagents: acidic anisaldehyde and NPR.

As compounds in the active F8a were present in the DCM fraction, this fraction was selected for further separation. 3.924g of the DCM fraction was reconstituted in DCM and mixed with silica gel ($60PF_{254}$), then ground in a mortar and allowed to dry overnight. The silica coated sample was applied to a Biotage SIM™ (35ml volume). Solvents of increasing polarity were added (500ml of each) under pressure (N_2: 5psi): PS (100%); PS : DCM (1:1); DCM (100%); DCM : MeOH (9:1); DCM : MeOH (6:1); DCM : MeOH (3:1); DCM : MeOH (1:1) and MeOH (100%); flow rate: $5ml.min^{-1}$. As solvent passed through the SIM™, the compounds from the sample were passed on to the Biotage prepacked column (i.d.: 15cm x 12mm, 8g). 70 fractions were collected and monitored using TLC and similar fractions were pooled to give 17 fractions (F1b - F17b). Each fraction was evaporated using a rotary evaporator, then under N_2 at room temperature. Fractions were weighed and diluted

in 96% EtOH to give a final concentration of 10mg/ml. Fractions were stored at 4°C. Refer to 2.2.2.2 and 2.2.2.3 for results and discussion.

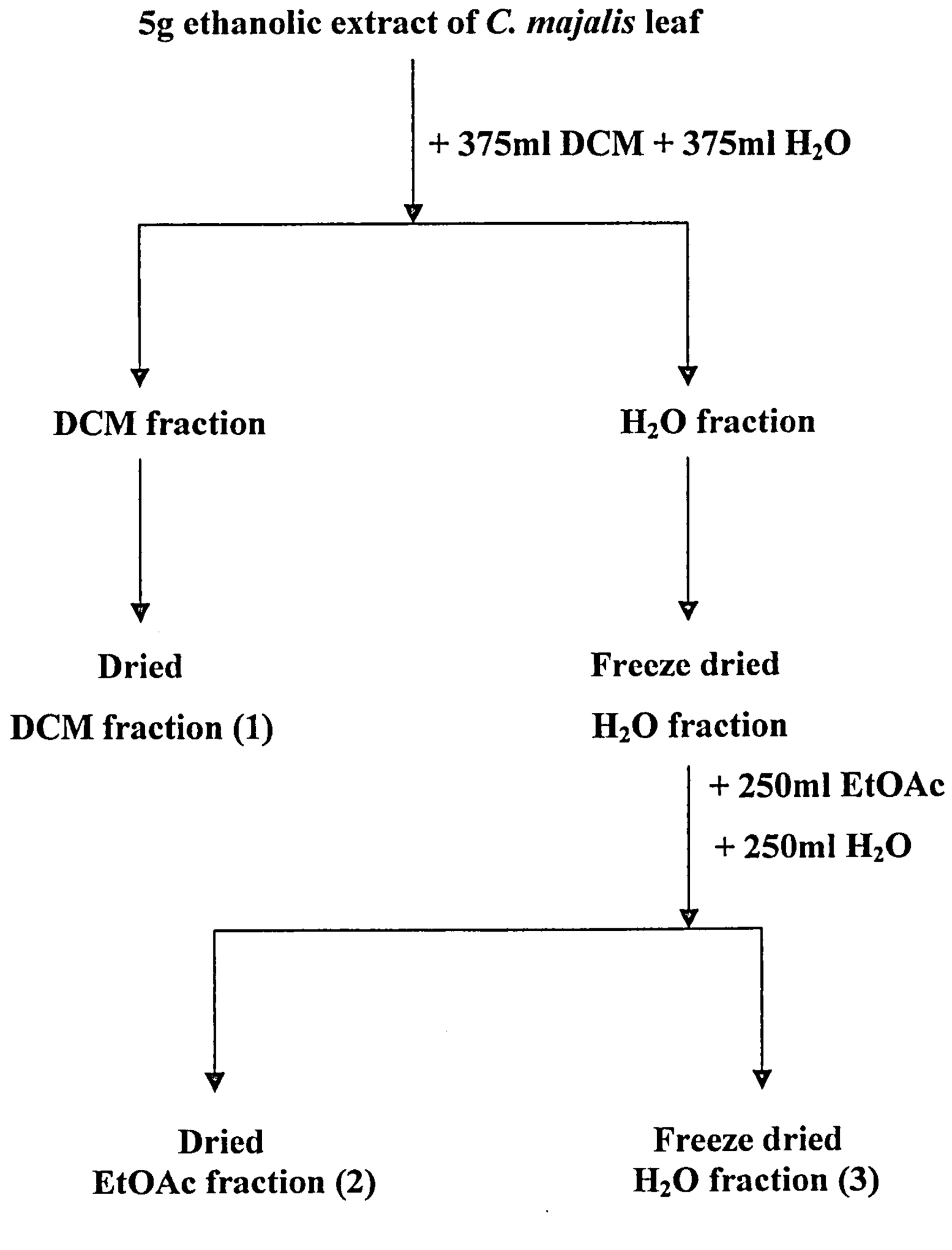


Figure 2.1. Diagram to show the partition of the ethanolic extract of *C. majalis* leaf.

2.1.6.3 Droplet Counter-Current Chromatography of *C. majalis* Leaf Extract

Liquid-liquid separation of a concentrated extract of *C. majalis* leaf (ethanolic extraction at room temperature) was performed with reference to the DCCC conditions discussed by Hostettmann *et al.* (1986). The Büchi-670 DCCC employed for separation was constructed with 290 silica glass vertical columns of 2.7mm diameter, each connected by capillary Teflon® tubes in a series of 6 racks. Parameters were set as follows: flow rate: 1ml.min^{-1}, maximum pressure: 30 bar and motor pump: 12 - 16. MeOH (100%) was passed through the system to prepare for addition of the stationary and mobile phases. The two-phase solvent system used for separation was DCM : MeOH : H_2O (5:6:4). The upper (stationary) phase was passed through the system. 3.219g of *C. majalis* leaf EtOH extract was dissolved in 20ml of the solvent system (1:1 of upper and lower solvent phases), and filtered prior to addition to the sample chamber. The lower less polar (mobile) phase was passed through the series of columns, displacing the extract from the sample chamber to form a series of descending droplets. 459 fractions (2ml - 3ml each fraction) were collected using a Retriever II (ISCO) fraction collector. Fractions were monitored using TLC and similar fractions pooled to give 17 final fractions, which were evaporated using a rotary evaporator, then under N_2 at room temperature. Fractions were weighed and diluted in 96% EtOH to give a final concentration of 10mg/ml. Fractions were stored at 4°C. Refer to 2.2.2.4 for results and discussion.

2.1.7 Analysis of *C. majalis* Leaf: Thin Layer Chromatography Methods

2.1.7.1 TLC Analysis of *C. majalis* Leaf Extracts Obtained by Soxhlet Extraction

5µl - 10µl applications of each of the extracts obtained by soxhlet extraction (C_6H_{14}, DCM, H_2O; refer to 2.1.3.4), and the crude EtOH extract of *C. majalis* leaf, were developed on silica gel plates ($60F_{254}$) using the following solvent systems: the lower less polar phase of the solvent system DCM : MeOH : H_2O (5:6:4), and $C_6H_5CH_3$: EtOAc : MeOH (5:6:4). Plates were examined under UV light (at 254nm and at 366nm), and in daylight after spraying with acidic anisaldehyde reagent. Refer to 2.2.4 for results and discussion.

2.1.7.2 TLC Analysis of Flash Column Chromatography Fractions of *C. majalis* Leaf

(a)

10µl - 20µl applications of each sample (fractions and reference sample of the unfractionated extract) were applied to the silica gel plate ($60F_{254}$) and plates were developed using different solvent systems to establish the most appropriate solvent system: $CHCl_3$ (100%); $C_6H_5CH_3$ (100%); $C_6H_5CH_3$: EtOAc (9:1); $C_6H_5CH_3$: EtOAc (3:1); DCM (100%), then after drying, developed again with EtOAc : MeOH (19:1) to approximately half the length of the solvent front; DCM (100%), then after drying, developed again with EtOAc : MeOH (1:1) to approximately half the length of the solvent front. The latter solvent system was selected for TLC analysis of the fractions. Every 3rd - 5th fraction was applied to the plate and developed using the selected solvent system. Fractions of higher polarity (F39 - F55) were also analysed using the solvent systems: EtOAc : MeOH (1:1) and EtOAc : MeOH : H_2O (100:13.5:1). All plates were examined under UV light (at 254nm and at 366nm), and in daylight after spraying with acidic anisaldehyde, followed by respraying with concentrated H_2SO_4 : H_2O (1:1) and reheating at 110°C for 5min (unless otherwise stated).

Fractions were also analysed using the solvent systems EtOAc : glacial CH_3COOH : CHOOH : H_2O (100:11:11:26) and the upper phase of butan-1-ol : glacial CH_3COOH : H_2O (4:1:5) and analysed using NPR.

Fractions which demonstrated antiChE activity were also analysed for presence of cardenolides: 10µl applications of fractions and reference samples (convallatoxin (**50**), cymarin (**48**) and the unfractionated extract) were applied to a silica gel plates ($60F_{254}$) and developed using the lower less polar phase of the solvent system DCM : MeOH : H_2O (5:6:4). Plates were analysed with Kedde's reagent, and also with chloramine T/trichloroacetic acid. Refer to 2.2.2.1 and 2.2.2.3 for results and discussion.

2.1.7.3 TLC Analysis of Flash Column Chromatography Fractions of *C. majalis* Leaf (b)

Every 5th fraction of the total of 70 fractions was analysed as follows: 10µl - 20µl applications of each sample (fractions and reference samples of the unfractionated extract, and F8a from FCC (a)) were applied to the silica gel plate ($60F_{254}$), and developed using the solvent system $C_6H_5CH_3$: EtOAc : MeOH (5:6:4) (e.g. F23 - F49, Figure 2.2).

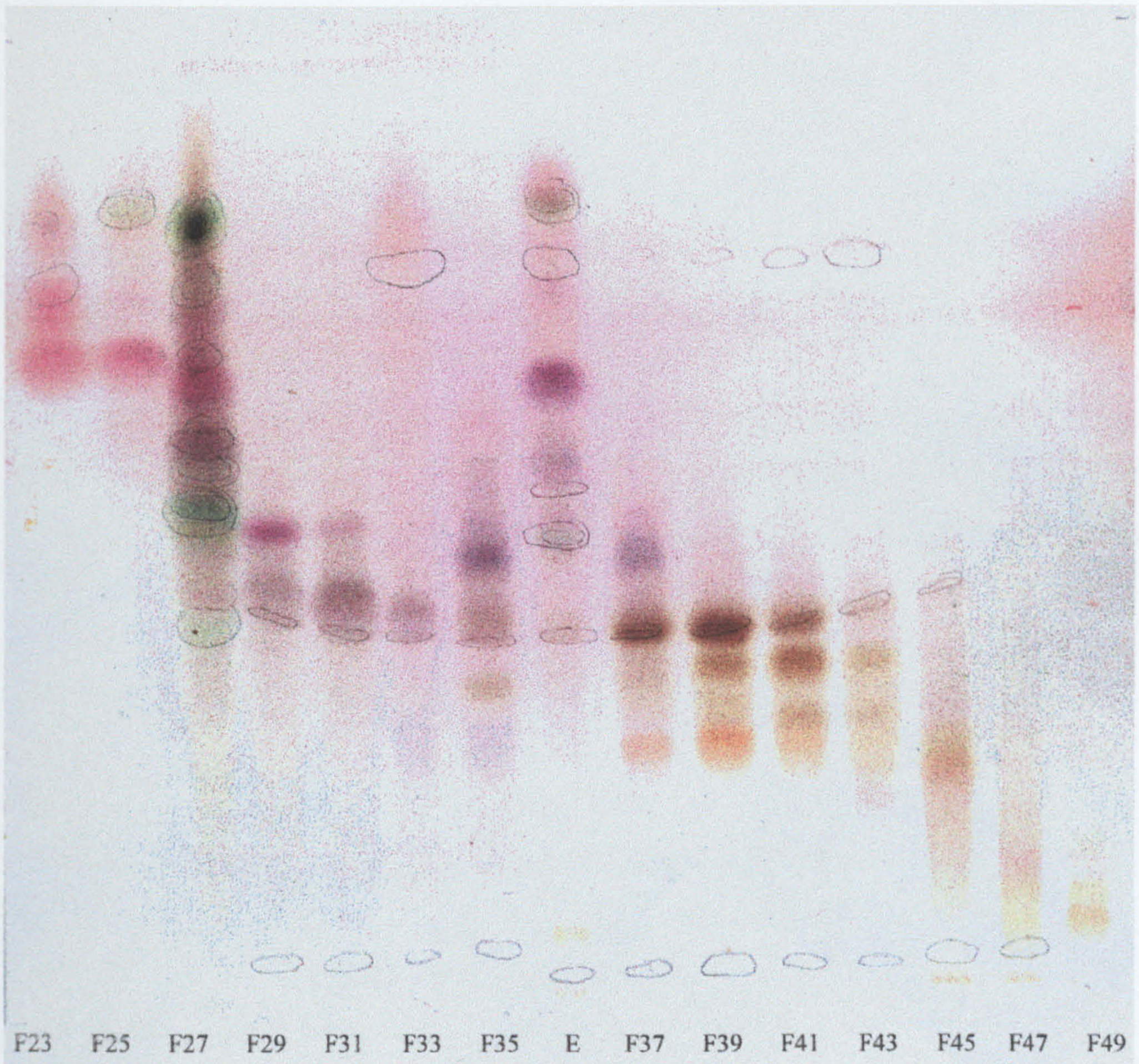


Figure 2.2. TLC profiles of flash column chromatography (b) fractions (F23 - F49) from *C. majalis* leaf extract. Chromatography conditions: stationary phase: silica gel ($60F_{254}$), mobile phase: $C_6H_5CH_3$: EtOAc : MeOH (5:6:4), spray reagent: acidic anisaldehyde. E = DCM fraction of EtOH extract of *C. majalis* leaf.

Fractions of greater polarity were also analysed using the solvent systems $C_6H_5CH_3$: EtOAc : MeOH (3:5:8) and EtOAc : MeOH : H_2O (100:13.5:10).
Fractions of lower polarity were also analysed using the solvent systems $C_6H_5CH_3$: EtOAc (93:7), $C_6H_5CH_3$: $CHCl_3$ (1:1), $C_6H_5CH_3$: $CHCl_3$ (4:1), C_6H_{14} : $CHCl_3$ (4:1) and C_6H_{14} : $CHCl_3$ (3:2). All plates were examined under UV light (at 254nm and at 366nm), and in daylight after spraying with acidic anisaldehyde reagent. Refer to 2.2.2.2 and 2.2.2.3 for results and discussion.

2.1.7.4 TLC Analysis to Compare *C. majalis* Leaf FCC (a) and (b) Active Fractions

For comparison of FCC fractions, a solvent system of $C_6H_5CH_3$: EtOAc : MeOH (5:6:4) and a stationary phase of silica gel ($60F_{254}$) plates were used; plates were examined under UV light (at 254nm and at 366nm), and in daylight after spraying with acidic anisaldehyde reagent. The crude EtOH *C. majalis* leaf extract and the DCM layer of the crude EtOH extract (prepared as described previously: 2.1.6.2 FCC (b)) were used as reference solutions.
The TLC profiles of F5b, F6b, F7b, F8b, F9b, F10b and F11b (from FCC (b)) were compared with F8a (from FCC (a)), and the TLC profiles of F10b and F11b (from FCC (b)) were compared with F15a and F16a (from FCC (a)). Refer to 2.2.2.3 for results and discussion.

2.1.7.5 Comparison of Crude Ethanolic Extract of *C. majalis* Leaf with Chlorophyll

5µl aliquots of the EtOH extract of *C. majalis* leaf, and chlorophyll (dissolved in H_2O, 1mg/ml) were applied to two silica gel ($60F_{254}$) plates; solvent systems DCM : MeOH (9:1) and DCM : MeOH (1:1) were used, respectively. Plates were examined under UV light (at 254nm and at 366nm), and in daylight after spraying with acidic anisaldehyde reagent. Refer to 2.2.5 for results and discussion.

2.1.7.6 Investigation to Establish an Appropriate Solvent System for Separation of *C. majalis* Leaf Extract Using DCCC

A crude *C. majalis* leaf EtOH extract was applied to both cellulose (prepared as described previously: 2.1.5.1) and silica gel ($60F_{254}$) plates. Plates were developed

using the upper and lower phases of the solvent systems DCM : MeOH : H_2O (5:6:4) and $CHCl_3$: MeOH : ^nPrOH : H_2O (5:6:1:4) (Hostettmann *et al.,* 1986). All silica gel plates were examined under UV light (at 254nm and at 366nm), and all plates were examined in daylight after spraying with acidic anisaldehyde reagent. Refer to 2.2.2.4 for results and discussion.

2.1.7.7 TLC Analysis of DCCC Fractions of *C. majalis* Leaf

Every 6th DCCC fraction was applied to silica gel ($60F_{254}$) plates and developed using the lower less polar phase of the solvent system: DCM : MeOH : H_2O (5:6:4) (e.g. F295 - F395, Figure 2.3).

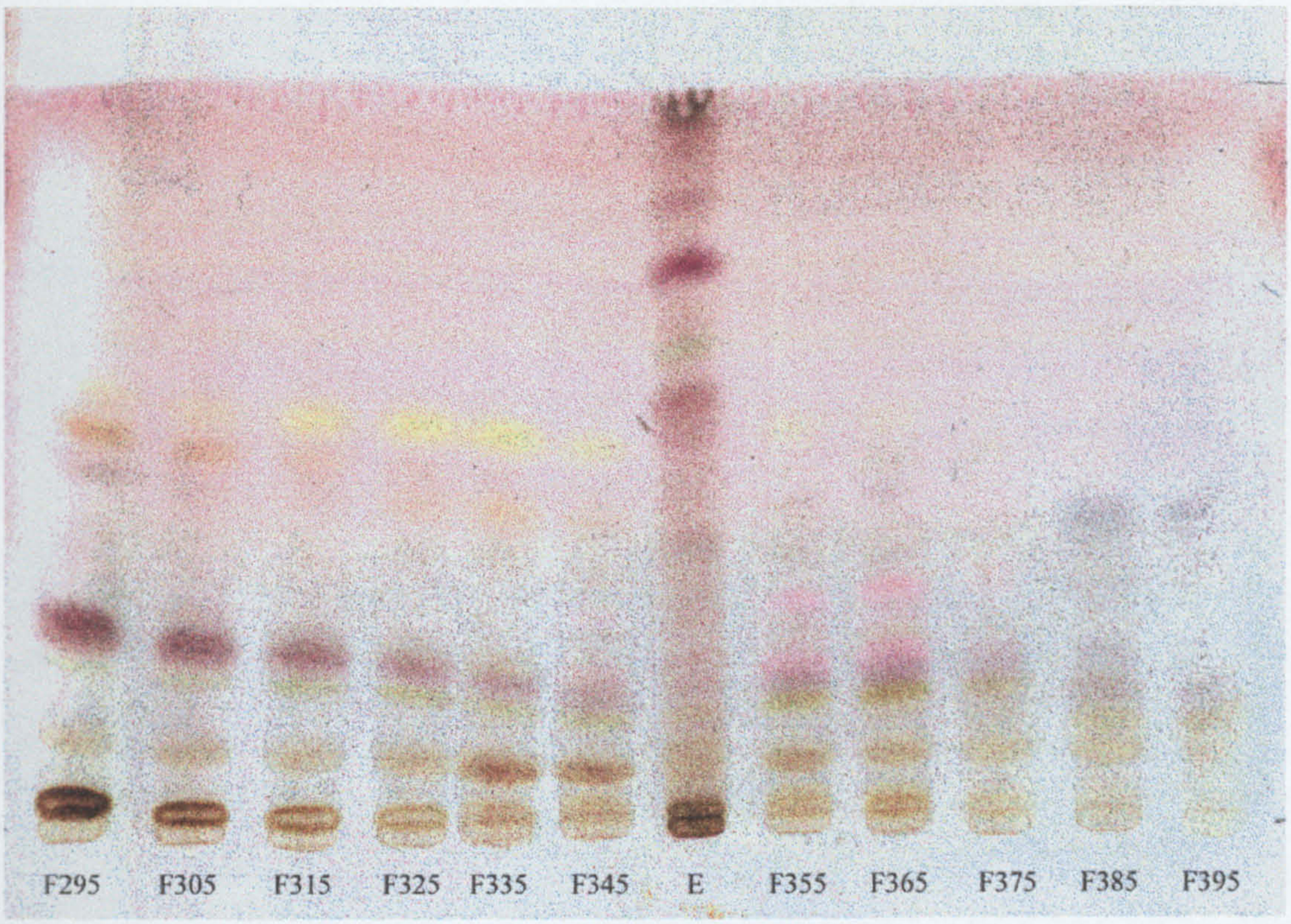


Figure 2.3. TLC profiles of droplet counter-current chromatography fractions (F295 - F395) from *C. majalis* leaf extract. Chromatography conditions: stationary phase: silica gel ($60F_{254}$), mobile phase: less polar phase of DCM : MeOH : H_2O (5:6:4), spray reagent: acidic anisaldehyde. E = crude EtOH extract of *C. majalis* leaf.

Fractions of greater polarity were also analysed using the upper, more polar phase of this solvent system, and also the solvent system EtOAc : MeOH : H_2O (100:13.5:10). The unfractionated crude extract was applied to each plate for reference. Plates were examined under UV light (at 254nm and at 366nm), and in daylight after spraying with acidic anisaldehyde reagent. F4 (obtained by pooling F62 - F71 from the original 459 DCCC fractions) was compared with F8a (from FCC(a)), and preparative TLC F3 and F4, using the lower less polar phase of the solvent system: DCM : MeOH : H_2O (5:6:4), and was examined under UV light (at 254nm and at 366nm), and in daylight after spraying with acidic anisaldehyde reagent. Refer to 2.2.2.4 for results and discussion.

2.1.7.8 Preparative TLC: *C. majalis* Leaf Ethanol Extract

40mg of *C. majalis* leaf crude EtOH extract, dissolved in 2ml 96% EtOH, was applied to one silica gel plate (prepared as described previously: 2.1.5.2) and dried at room temperature. The plate was developed using the lower less polar phase of the solvent system DCM : MeOH : H_2O (5:6:4), dried, and a small section of the plate sprayed with acidic anisaldehyde reagent. The plate was also analysed under ultraviolet light (at 254nm and at 366nm). The plate was divided into 7 sections (including the baseline) and each section (excluding the sprayed section) was scraped off and dissolved in 20ml - 40ml DCM : MeOH (1:1). Each of the 7 fractions were filtered to remove silica, evaporated to dryness by blowing with N_2 at room temperature and dissolved in 96% EtOH to give a 2mg/ml concentration. Fractions diluted in EtOH were stored at 4°C.

Each of the 7 fractions was analysed by application to silica gel ($60F_{254}$) plates, and developed using the solvent system $C_6H_5CH_3$: EtOAc : MeOH (5:6:4). The plate was examined under UV light (at 254nm and at 366nm), and in daylight after spraying with acidic anisaldehyde reagent.

F3 and F4 (which inhibited AChE, refer to Chapter 3, 3.2.4.5) were also compared with the crude EtOH extract of *C. majalis* leaf, and with the active F8a (from FCC (a)) by application to silica gel ($60F_{254}$) plates. The solvent systems used for analysis were the lower less polar phase of the solvent system DCM : MeOH : H_2O (5:6:4), and $C_6H_5CH_3$: EtOAc : MeOH (5:6:4). Plates were examined under UV light (at

and at 366nm), and in daylight after spraying with acidic anisaldehyde reagent. Plates were also examined for the presence of alkaloids using Dragendorff's spray reagent, instead of acidic anisaldehyde. Refer to 2.2.2.5 for results and discussion.

2.1.8 Analysis of *C. majalis* Leaf Extract and Fractions Using HPLC and LC-MS

2.1.8.1 Analysis of *C. majalis* Leaf Crude Ethanolic Extract and Preparative TLC Fractions

LC-MS investigations were conducted by Dr Geoffrey Kite, The Royal Botanic Gardens, Kew, England and HPLC analysis of F3 (from preparative TLC separation of *C. majalis* leaf EtOH extract, refer to 2.1.7.8) was conducted by Dr Renee Grayer, The Royal Botanic Gardens, Kew, England.

LC-MS analysis of the crude EtOH extract and the preparative TLC fractions (F1 - F7) of *C. majalis* leaf, was conducted as follows:

15mg of dried EtOH extract was dissolved in 2ml MeOH; dried fractions (F1 - F7) were also dissolved in MeOH. HPLC parameters were as follows: column: C18 (Supelco Discovery), 250mm x 4.6mm, 5µm; pump: Waters 600E; UV detector: L-4000 (Merck Hitachi); wavelength: 210nm; solvent gradient (for crude extract): 70% H_2O, 20% MeOH, 5% C_2H_3N (t=0min), 0% H_2O, 95% MeOH, 5% C_2H_3N (t=25min), solvent gradient (for fractions): 60% H_2O, 35% MeOH, 5% C_2H_3N (t=0min), 0% H_2O, 95% MeOH, 5% C_2H_3N (t=25min), flow rate: $1ml.min^{-1}$.
MS parameters were as follows: MS: quadruple ion-trap (Finnigan MAT, model LCQ); source: APCI operating in positive mode; vaporisation temperature: 450°C; N_2 pressures: 80psi (sheath), 20psi (auxillary); needle voltage: 4.2kV. The MS was set to record a survey scan in the range *m/z* 100 - 2000, and then the CID spectrum of the most intense ion in the survey scan. Refer to 2.2.3 for results and discussion.

HPLC analysis of preparative TLC F3 from the EtOH extract of *C. majalis* leaf, was conducted as follows:

Column: Lichrosphere 100RP, 250mm x 4mm; column temperature: 30°C; pump: Waters LC 600; UV detector: L-4000 (Merck Hitachi); wavelength: 257nm; solvent gradient: 75% A, 25% B (t=0min), 0% A, 100% B (t=20min), followed by isocratic elution to t=23min (where solvent A: 2% CH_3COOH in distilled H_2O; solvent B: MeOH : H_2O : CH_3COOH (18:1:1)), flow rate: 1ml.min^{-1}. Refer to 2.2.3 for results and discussion.

2.1.8.2 LC-MS Analysis of *C. majalis* Leaf Flash Column Chromatography Fractions (FCC (b))

LC-MS analysis of F6b and F7b (from FCC (b)) was conducted by Dr Geoffrey Kite, The Royal Botanic Gardens, Kew, England.

LC-MS analysis of F6b and F7b (from FCC (b)) of *C. majalis* leaf extract was conducted as follows:

15mg of each fraction was dissolved in 2ml MeOH. HPLC parameters were as follows: column: C18 (Merck LiChrosphere), 250mm x 4mm (particle size: 5µm); pump: Waters 600E; UV detector: L-4000 (Merck Hitachi); wavelength: 335nm; solvent gradient: 25% - 100% MeOH in 1% CH_3COOH in 20min, flow rate: 1ml.min^{-1}. MS parameters were as follows: MS: quadruple ion-trap (Finnigan MAT, model LCQ); source: APCI operating in positive mode; vaporisation temperature: 450°C; N_2 pressures: 80psi (sheath), 20psi (auxillary); needle voltage: 4.2kV. The MS was set to record a survey scan in the range *m/z* 100 - 2000, and then the CID spectrum of the most intense ion in the survey scan. Refer to 2.2.3 for results and discussion.

2.1.9 Analysis of Herbal Extracts for the Presence of GABA

All aqueous and ethanolic extracts of each plant were analysed for the presence of GABA (**16**). This analysis was performed to enable exclusion of all GABA (**16**)-containing extracts from the GABA (**16**) receptor binding assay; presence of GABA (**16**) in an extract would interfere in this assay by giving false positive results.
20µl applications (equivalent to 100µg of herbal extract) were applied to cellulose plates (prepared as described in 2.1.5.1), and also to silica gel ($60F_{254}$) plates. 5µl GABA (**16**) (20nmoles in distilled water) was applied to each plate for reference. Plates were developed using the solvent system butan-1-ol : H_2O : CH_3COOH (60:25:15), and sprayed with ninhydrin reagent. Refer to 2.2.6 for results and discussion.

2.1.10 Gas Chromatography-Mass Spectroscopy

<u>2.1.10.1 Analysis of *M. officinalis* Essential Oil and Commercially Obtained Oil Constituents</u>

GC-MS investigations were conducted in collaboration with Dr Geoffrey Kite, The Royal Botanic Gardens, Kew, England.

GC-MS analysis was conducted using a GC (Perkin-Elmer 8500) with parameters set as follows: oven temperature: 60°C - 200°C (3°C.minute^{-1}), carrier gas: He (20psi). The column used was a capillary column, length: 25m x 0.22mm i.d., coating: 0.25µm of BPX5 (SGE Ltd., 1 Potters Lane, Kiln Farm, Milton Keynes, MK11 34X). The MS was a Finnigan Ion Trap Detector, 400 series, scanning: *m/z* 38 - 300. The injection volume for all samples was 1µl, split.

The major components in the oils were identified by comparing mass spectra with published data (Adams, 1995) and calculating KI values. The identifications of citral (geranial (**70**) and neral (**71**)), geraniol (72) and linalool (**74**) were confirmed by comparison with standards. Percentage compositions were calculated from both the peak areas in the total ion chromatogram (TIC) and the peak areas in the flame ionisation detection (FID) trace.

Samples analysed were as follows:

Sample Oil/Compound	Source	Dilution
Melissa officinalis	Fragrant Earth	1/100 in C_6H_{14}
Melissa officinalis phytol extract	Clwydian Fragrant Oil	1/100 in $(C_2H_5)_2O$
Melissa officinalis phytol (crude extract)	Clwydian Fragrant Oil	1/100 in $(C_2H_5)_2O$
Citral (95%)	Aldrich	1/1000 in C_6H_{14}
Citral	Lancaster	1/1000 in C_6H_{14}
Geraniol (98%)	Sigma	1/1000 in C_6H_{14}
(±)-Linalool (97%)	Aldrich	1/1000 in C_6H_{14}
Nerol (98%)	Sigma	1/1000 in C_6H_{14}

2.1.10.2 Assessment of Metabolism of Compounds by Yeast

GC-MS investigations were conducted with Dr Geoffrey Kite, The Royal Botanic Gardens, Kew, England.

To determine if metabolism of the four compounds (citral, eugenol (**84**), geraniol (**70**) and nerol (**75**)) by the yeast cells occurred, each compound was incubated in the presence and absence of yeast, and the results compared using GC-MS analysis (refer to Chapter 4, 4.1.5, 4.7.2 and 4.10.2).

The $(C_2H_5)_2O$ phase from each glass vessel, containing yeast suspension and $(C_2H_5)_2O$, was removed and stored in a sealed glass vessel at 4°C.

The vessel contents were analysed using a Perkin Elmer model 8500 GC; column: 25m x 0.2mm i.d., coating: 0.25µm BPX5 (SGE Ltd., 1 Potters Lane, Kiln Farm, Milton Keynes, MK11 34X); carrier gas: He (20psi); oven programme: 80°C - 200°C (5°C.min^{-1}); detection: FID and ITD (Finnigan-MAT) with ITD parameters of *m/z* 38 - 400 scan range, AGC background mass 38, 1 scan.s^{-1}, and an injection volume of 1µl, split.

2.2 Results and Discussion

2.2.1 Authentication of Plant Material

2.2.1.1 Authentication of *Polygonum multiflorum* Root

Morphological analysis of the *P. multiflorum* root sample, compared with a sample of authentic plant material, indicated that the sample was not *P. multiflorum* root (Figures 2.4 and 2.5).

Figure 2.4. *Polygonum multiflorum* root.

Figure 2.5. Sample claimed to be *Polygonum multiflorum* root in the present study.

To investigate this further, the *P. multiflorum* root sample was compared with *Gentiana scabra* root, a herb which was morphologically similar to the *P. multiflorum* root sample, using HPLC analysis. The chromatograms for each herb were similar, although not identical (refer to Table 2.1 and appendix, Figures A1 and A2). The major component of both extracts gave a peak in the chromatogram at the retention time of 6.4min, however the maximum absorbance in the UV spectrum of this peak was too high in the *Gentiana scabra* root chromatogram, due to the high concentration of the compound giving this peak, to accurately determine the maximum absorbance (refer to appendix, Figures A1 and A2). The peaks in both chromatograms with retention times from 15.3min - 19.7min gave similar UV spectra, suggesting the compounds responsible for these peaks are chemically related (refer to appendix, Figures A1 and A2).

Table 2.1. Retention times (min) of peaks in the chromatograms of *Gentiana scabra* root (authentic) and *Polygonum multiflorum* root (sample) using HPLC analysis.

***Gentiana scabra* Root (Authentic)**	***Polygonum multiflorum* Root (Sample)**
2.72min	2.72min
6.42min	6.43min
7.21min	7.26min
16.02min	16.04min
17.23min	17.23min
19.41min	19.41min

In view of the morphological similarities and the similar chromatograms given by both extracts, it may be concluded that the *P. multiflorum* root sample is likely to be a *Gentiana* spp., but does not appear to be *Gentiana scabra* as there were some differences between the chromatograms (refer to appendix, Figures A1 and A2). Further analysis of the *P. multiflorum* root sample is required to confirm which *Gentiana* spp. it is. Therefore, the *P. multiflorum* root sample will be described as 'adulterated *P. multiflorum* root'.

2.2.1.2 Authentication of *Rosmarinus officinalis* Fresh Leaf

Analysis of the 80% MeOH and the $(C_2H_5)_2O$ extracts of *Rosmarinus officinalis* fresh leaf using HPLC indicated that the *R. officinalis* leaf sample was the correct species. The corresponding chromatograms for the sample and the authentic plant material were similar but not identical (refer to appendix, Figures A3, A4, A5, A6, A7 and A8).

Differences observed between the chromatograms may have occurred due to natural variation in *R. officinalis* plants (e.g. due to cultivation conditions or time of harvesting). The *R. officinalis* leaf sample was frozen prior to HPLC and GC-MS analysis, which may also have influenced the chemical composition, but the authentic *R. officinalis* leaf was not frozen. The UV spectra of the peaks in the chromatograms yielded information regarding the chemical composition of the sample and authentic *R. officinalis* plants (Tables 2.2 and 2.3). The phytochemistry of the authentic plant material was similar to that of the *R. officinalis* sample (Tables 2.2 and 2.3 and appendix, Figures A3, A4, A5 and A6) and in view of the differences in preparation of the plant material prior to analysis, indicates that the sample is *R. officinalis*.

Analysis of the essential oil composition of both the sample and authentic *R. officinalis* plants was conducted to provide further information regarding the authenticity of the *R. officinalis* sample. The essential oil compositions of the plants are different (refer to Table 2.4 and appendix, Figures A7 and A8), which may be explained by the cultivation conditions, by the plants being different varieties or at different stages of development, or the freezing process of the *R. officinalis* sample. There are some essential oil components common to both of the *R. officinalis* essential oils, although their percentage composition does differ.

Table 2.2. Retention times (min) of peaks in the chromatograms of *Rosmarinus officinalis* leaf (sample and authentic) methanol extracts using HPLC analysis.

***R. officinalis* Leaf (Authentic): R_t (min)**	***R. officinalis* Leaf (Sample): R_t (min)**	**Proposed Chemistry (Based on UV Spectra)**
3.62	-	caffeic acid derivative
4.09	-	caffeic acid derivative
9.80	-	flavone glycoside
11.69	-	unidentified
11.92	-	unidentified
12.20	-	unidentified
12.39	-	unidentified
12.70	12.73	flavone glycoside
12.96	-	unidentified
13.26	-	rosmarinic acid
-	13.73	unidentified
13.93	-	unidentified
14.34	14.34	flavone glycoside
14.61	-	unidentified
15.20	15.2	flavone glycoside
15.72	-	flavone glycoside
16.22	-	flavone glycoside
16.51	-	unidentified
16.87	16.87	flavone glycoside
-	17.17	unidentified
24.33	24.30	phenylpropanoid
26.80	26.80	phenylpropanoid
27.57	27.57	phenylpropanoid

Table 2.3. Retention times (min) of peaks in the chromatograms of *Rosmarinus officinalis* leaf (sample and authentic) diethyl ether extracts using HPLC analysis.

***R. officinalis* Leaf (Authentic): R_t (min)**	***R. officinalis* Leaf (Sample): R_t (min)**	**Proposed Chemistry (Based on UV Spectra)**
4.64	4.65	unidentified
-	5.62	phenolic acid
-	6.45	phenolic acid
-	8.57	unidentified
-	10.24	unidentified
-	12.74	flavone glycoside
13.35	-	rosmarinic acid
13.73	13.73	unidentified
14.35	14.34	flavone glycoside
-	16.87	unidentified
16.90	-	flavone glycoside
-	17.13	unidentified
17.17	-	phenylpropanoid
-	17.49	unidentified
17.52	-	caffeic acid derivative
18.16	-	caffeic acid derivative
-	18.31	unidentified
-	19.01	flavone aglycone
20.28	20.27	flavone aglycone
-	22.95	flavone aglycone
23.41	23.41	flavone aglycone
24.34	24.30	phenylpropanoid
25.18	25.15	diterpene
26.10	-	unidentified
-	26.80	phenylpropanoid
27.57	27.52	phenylpropanoid

Table 2.4. Percentage composition of *Rosmarinus officinalis* leaf essential oils obtained from a sample and from authentic plant material, determined by desorption-GC-MS analysis.

Component	**R_t (min)**	***R. officinalis* (Sample)**	***R. officinalis* (Authentic)**
tricyclene	9.4	<0.1	0.3
α-pinene	10.0	4.2	21.3
camphene	10.6	2.3	6.3
thuja-2, 4(10)-diene	10.8	<0.1	0.4
β-pinene	11.8	2.4	2.2
3-octanone	12.4	<0.1	1.0
myrcene	12.5	8.0	3.9
unidentified	13.0	1.8	0.4
α-terpinene	13.5	0.5	0.6
o-cymene	13.9	0.5	0.4
limonene	14.1	4.5	4.5
1, 8-cineole	14.2	18.5	9.5
γ-terpinene	15.3	1.0	0.7
unidentified	15.8	0.6	0.3
terpinolene	16.4	0.6	0.7
unidentified	17.0	<0.1	0.3
linalool	17.2	1.0	1.3
chrysanthenone	17.9	0.3	2.0
camphor	19.0	23.1	10.4
unidentified	19.4	<0.1	0.7
borneol	20.0	7.3	7.6
unidentified	20.3	0.8	0.6
α-terpineol	21.0	2.6	1.1
verbenone	21.4	11.2	11.9
unidentified	22.5	<0.1	0.9
unidentified	22.8	<0.1	1.3
unidentified	23.6	<0.1	0.5
bornyl acetate	24.1	1.9	1.2
piperitenone	26.0	<0.1	0.4
caryophyllene	28.8	2.2	4.8
unidentified	29.8	<0.1	0.2
α-humulene	30.0	4.2	0.8
caryophyllene oxide	34.0	0.3	0.7
unidentified	35.7	<0.1	0.2
unidentified	36.3	0.3	0.7

The major constituents reported to be present in *R. officinalis* essential oil are 1, 8-cineole (**56**) (15-30%), camphor (**57**) (15-25%), α-pinene (**53**) (up to 25%) and other monoterpenes including borneol (**58**) and limonene (**73**) (Bisset, 1994; Trease and Evans, 1996). 1, 8-Cineole (**56**) and camphor (**57**) composed 18.5% and 23.1% respectively of the sample essential oil, but only 9.5% and 10.4% respectively of the authentic essential oil; however, α-pinene (**53**) was at a higher concentration in the

authentic essential oil (21.3%) than in the sample essential oil (4.2%) (refer to Table 2.4). Borneol (**58**) and limonene (**73**) were at similar concentrations in both essential oils. The similar composition of both essential oils and the presence of constituents reported to be present in *R. officinalis* essential oil, indicates that the sample of *R. officinalis* leaf is the correct species.

2.2.1.3 Authentication of *Withania somnifera* Root

The sample of *Withania somnifera* root was analysed using TLC, after spraying with 10% H_2SO_4 reagent in MeOH and examination in UV light (366nm) and also in daylight (refer to 2.1.2.3 for method). The TLC profile for the sample was similar to the TLC profile of authentic *W. somnifera* root, as described by Upton, 2000. Notably, in the TLC profile of the sample, there was a light blue fluorescent zone (R_f: 0.58) visible in UV light (366nm) that corresponds to β-sitosterol (**88**), and there was a pink-red zone (R_f: 0.17) visible in daylight that corresponds to withaferin A (**109**) (both compounds visible after spraying with the reagent) (Figure 2.6).

Figure 2.6. TLC profile of *Withania somnifera* root sample. Mobile phase: $C_6H_5CH_3$: EtOAc : CHOOH (50:15:5); spray reagent: 10% H_2SO_4 in MeOH. R_f: 0.17 corresponds to withaferin A; R_f: 0.58 corresponds to β-sitosterol.

The TLC profile, including the identification of the reference compounds β-sitosterol (**88**) and withaferin A (**109**), indicates that the sample of *W. somnifera* root used in the present study is the correct species.

2.2.2 Analysis of Separation Methods of *C. majalis* Leaf Extracts

2.2.2.1 Analysis of Flash Column Chromatography (a) Fractions of *C. majalis* Leaf Ethanolic Extract

Several solvent systems were investigated to establish the most efficient solvent system for analysis of the FCC fractions using TLC. The solvent systems $CHCl_3$ (100%) and $C_6H_5CH_3$ (100%) gave insufficient separation of compounds. These solvent systems were used to analyse fractions including F1 - F6 (of the total 55 fractions), which consisted of compounds of low polarity (C_6H_{14} fractions). Inadequate separation was observed following examination under UV light (at 254nm and at 366nm), and in daylight after spraying with acidic anisaldehyde. The solvent system $C_6H_5CH_3$: EtOAc (9:1) gave better separation of the less polar fractions (F1 - F6), however this system was unsuitable for analysis as the polarity of the compounds in the fractions increased. Increasing the polarity of the solvent system to achieve better separation was carried out by using a system of $C_6H_5CH_3$: EtOAc (3:1). Separation of F1 - F15 was improved, but this system was unsuitable for separation of F16 - F55. A disadvantage of the solvent systems $C_6H_5CH_3$: EtOAc (9:1 and 3:1) was the observation of trailing of some zones on the TLC plate. The most efficient separation was observed with the solvent system DCM (100%), and then after drying, with EtOAc : MeOH (1:1) to approximately half the length of the solvent front. This method of analysis was employed for F1 - F40, but did not give adequate separation for F41 - F55. F41 - F55 were analysed using the solvent systems EtOAc : MeOH (1:1) and EtOAc : MeOH : H_2O (100:13.5:1); systems suitable for the separation of more polar compounds such as the cardiac glycosides, reported to be present in *C. majalis* leaf (Bruneton, 1995).

The fractions and the crude EtOH extract, were also analysed using the solvent systems EtOAc : glacial CH_3COOH : CHOOH : H_2O (100:11:11:26) and the upper phase of butan-1-ol : glacial CH_3COOH : H_2O (4:1:5), and sprayed with NPR and analysed in daylight for detection of flavonoid compounds. The latter solvent system

gave good separation of compounds. An orange zone (R_f: 0.29) in the crude EtOH extract indicated that it may be a flavonoid other than rutin (R_f: 0.47) or hyperoside (R_f: 0.60). However, no other flavonoid compounds were detected in the fractions. The glycosidic forms of flavonoids may be present in the vacuoles of the epiderm and mesophyll of leaves, and would therefore be expected to occur in *C. majalis* leaf. Flavonoid aglycones that are more lipophilic are often present in the cuticle of leaves; however, these may be in low concentration or absent in the dried *C. majalis* leaf.

From TLC examination, the fractions (from the original 55 fractions) containing similar constituents were pooled to give 16 fractions. Fractions (some of those that inhibited AChE in the *in vitro* assay, refer to Chapter 3, 3.2.4.2) F3a - F5a, F8a - F9a and F13a - F16a, and also the crude EtOH extract, were analysed for the presence of cardenolide compounds. Analysis with Kedde's reagent did not demonstrate the presence of cardenolides in any of the fractions or crude extract, however the reference compounds (convallatoxin (**50**) and cymarin (**48**)) gave the characteristic purple colour zones. These fractions were also analysed with chloramine T/trichloroacetic acid to confirm the results; the reference compounds convallatoxin (**50**) (R_f: 0.30) and cymarin (**48**) (R_f: 0.52) gave blue-green fluorescence under 366nm UV light. In F3a, one zone (R_f: 0.73) was fluorescent blue under 366nm UV light before spraying, but was fluorescent green after spraying; and in F8a, one zone (R_f: 0.27) was fluorescent orange under 366nm UV light before spraying, and was fluorescent green after spraying. These results indicate that cardenolide compounds may be present in both F3a and F8a.

The negative results for cardenolides in the other fractions analysed may be due to the absence of such compounds, or due to their presence in a concentration too low for detection. The cardenolides present in F3a and F8a did not appear to be either convallatoxin (**50**) or cymarin (**48**), as the R_f values show. Convallatoxin (**50**) has been reported to be the major cardiac glycoside from the flowers and leaves of *C. majalis* (Harborne, 1993). It would therefore be reasonable to expect the detection of this compound in the crude extract or in one or more fractions of *C. majalis* leaf. This may be explained by differences in the chemical composition of *C. majalis* due to variation in cultivation conditions (e.g. climate, season, soil, and temperature), in harvesting processes, in storage conditions of the harvested plants, or differences in phenotype.

2.2.2.2 Analysis of Flash Column Chromatography (b) Fractions of *C. majalis* Leaf Extract (Obtained from the Dichloromethane Layer of an Ethanolic Extract)

The partition of the EtOH extract of *C. majalis* leaf between DCM and H_2O would have removed many of the more polar compounds present in the aqueous layer, which did not significantly inhibit AChE (refer to Chapter 3, 3.2.4.1), leaving the less polar compounds in the DCM layer. It is reported that some cardenolide glycosides may be water soluble, and others more readily soluble in $CHCl_3$ (Lugt, 1975). It is therefore concluded that some glycosides and the aglycones were present in the DCM fraction of the EtOH extract, while other glycosides would have been extracted into the aqueous fraction. The DCM fraction was selected for further separation, as compounds corresponding to those in the active F8a (from FCC (a)) were present.

The solvent system $C_6H_5CH_3$: EtOAc : MeOH (5:6:4) gave efficient separation of F10 - F43. The more polar fractions (F44 - F70) were also analysed using the solvent systems $C_6H_5CH_3$: EtOAc : MeOH (3:5:8) and EtOAc : MeOH : H_2O (100:13.5:10), the latter giving better separation for analysis. F1 - F9 were also analysed using several solvent systems; C_6H_{14} : $CHCl_3$ (4:1) was most suitable for F1 - F6, and $C_6H_5CH_3$: $CHCl_3$ (1:1) was most suitable for analysis of F7 - F9.

2.2.2.3 TLC Analysis to Compare FCC (a) and (b) Active Fractions

Fractions from both FCC separations ((a) and (b)), which showed activity in the antiChE assay, were analysed to identify any common constituents.

Six zones in F8a (from FCC (a)) were also present in F7b (from FCC (b)), one giving a green zone (R_f: 0.56) after spraying with acidic anisaldehyde, the others giving fluorescent orange zones (R_f: 0.4, R_f: 0.54, R_f: 0.59, R_f: 0.77, R_f: 0.85) when examined under UV light (366nm). Four of these zones (R_f: 0.54, R_f: 0.59, R_f: 0.77, R_f: 0.85) were also present in F6b, two zones (R_f: 0.4, R_f: 0.85) were also present in F8b, one zone was also present in F5b (R_f: 0.85), and one zone (R_f: 0.54) present in F9b from FCC (b).

These results suggest that a compound (or compounds) identified in both active fractions from both FCC (a) and (b) may be responsible for the antiChE activity observed in the *in vitro* assays. Alternatively, different compounds in the active fractions may have antiChE activity. This may explain the antiChE activity observed

with F15a and F16a (from FCC (a)) and F10b and F11b (from FCC (b)) (refer to Chapter 3, 3.2.4.2 and 3.2.4.3), as no compounds were identified to be present in both FCC (a) (F15a and F16a) and FCC (b) (F10b and F11b) fractions.

2.2.2.4 TLC Analysis of DCCC Fractions of *C. majalis* Leaf Ethanolic Extract

DCCC was selected for separation of the constituents of *C. majalis* leaf EtOH extract, as it has the advantage that no irreversible adsorption of the sample occurs during separation, due to the absence of a solid support (e.g. the stationary phase in FCC). Therefore, theoretically, there should be complete recovery of the sample. Separation of the constituents is achieved as the solute is partitioned between the stationary phase and the mobile phase (the droplets).

A DCCC separation method using the upper and lower phases of the solvent systems DCM : MeOH : H_2O (5:6:4) and $CHCl_3$: MeOH : H_2O (5:6:4) has been used for the isolation of cardiac glycosides from *Digitalis lanata* leaf (Krüger *et al.*, 1983); strophanthidin glycosides have been isolated from the arrow poison *Lophopetalum toxicum* using the solvent systems of $CHCl_3$: MeOH : $^{n}PrOH$: H_2O (5:6:1:4) and $CHCl_3$: MeOH : $^{n}PrOH$: H_2O (45:70:5:40) in a DCCC system (Hostettmann *et al.*, 1986). These solvent systems were selected to assess the most appropriate system for separation of the compounds in the EtOH extract of *C. majalis* leaf. Ternary solvent systems were selected to influence droplet size and achieve good separation of compounds. A binary solvent system is not suitable for this, as the large difference in polarity between the two solvents would not achieve suitable droplets for the separation of similar compounds; addition of a third solvent minimises the differences in polarity of the system. Solvent systems composed of chlorinated components have high densities and low viscosities, and are reported to be suitable for use as the mobile phase in DCCC separations, and in combination with H_2O and MeOH give good formation of droplets (Hostettmann *et al.*, 1984). Solvent systems composed of $CHCl_3$ (or DCM) : MeOH : H_2O in varying proportions have been employed for the successful isolation of several types of compound, including glycosides, tannins, alkaloids, saponins, xanthones, lignans and diterpenoids (Hostettmann, 1980; Hostettmann *et al.*, 1984). Therefore the use of solvent systems DCM : MeOH : H_2O (5:6:4) and $CHCl_3$: MeOH : H_2O (5:6:4), suggests there is potential for the isolation of cardiac glycosides and other compounds present in *C. majalis* leaf.

The crude EtOH extract of *C. majalis* leaf was examined by TLC using the less polar phase of the two solvent systems as the mobile phase, and silica gel ($60F_{254}$) for the stationary phase. The R_f values of the zones dictate which of the two solvent phases are appropriate for the mobile and stationary phases in the DCCC system. The less polar phase of the solvent system $CHCl_3$: MeOH : H_2O (5:6:4) resulted in 4 zones with R_f values <0.5 and 6 zones with R_f values >0.5 (Table 2.6, Figure 2.8). This suggests that the compounds separated are relatively non-polar, therefore the less polar phase of the solvent system would be favoured for the mobile phase. The less polar phase of the solvent system $CHCl_3$: MeOH : ^nPrOH : H_2O (5:6:1:4) resulted in 7 zones with R_f values <0.5 and 4 zones with R_f values >0.5 (Table 2.7, Figure 2.9). It would be more appropriate in this case to select the polar phase of the solvent system for the mobile phase, as the compounds separated are of higher polarity.

Analysis of the R_f values with the less polar phases of the two solvent systems DCM : MeOH : H_2O (5:6:4) and $CHCl_3$: MeOH : ^nPrOH : H_2O (45:70:5:40) showed 5 zones with R_f values >0.5, and 5 zones with R_f values <0.5 (Tables 2.5 and 2.8, Figures 2.7 and 2.10). Consequently, either phase of the solvent system may be selected for the mobile phase. The solvent system DCM : MeOH : H_2O (5:6:4) was selected for separation using DCCC, as this system gave more efficient separation of the compounds than with the other three solvent systems. The more lipophilic phase was used as the mobile phase and the polar phase used as the stationary phase (descending mode).

The DCCC F4 (obtained by pooling fractions 62 - 71 from the original 459 DCCC fractions) was compared with the F8a (from FCC(a)), and preparative TLC F3 and F4. The zones which were identified in F8a (from FCC(a)), and preparative TLC F3 and F4 (see below: *, ** and ***, Figure 2.13 and Tables 2.9 and 2.10) were not identified in DCCC F4. Five of the ten zones in DCCC F4 had low R_f values (R_f values: 0.03, 0.12, 0.19, 0.36, 0.43) but other zones present had higher R_f values (R_f values: 0.56, 0.65, 0.73, 0.83, 0.98). This indicates that both polar and more lipophilic compounds are present in this fraction.

Table 2.5. R_f values of zones from *C. majalis* leaf ethanolic extract (corresponding to Figure 2.7), examined in daylight (detection using acidic anisaldehyde, * examination under 366nm UV light).

Zone on TLC Plate	**R_f value**
Brown	0.01
Brown/Yellow	0.05
Pink	0.16
Pink	0.24
Pink	0.47
Green (Orange*)	0.54
Deep Pink	0.62
Pink	0.68
Pink	0.84
Green (Orange*)	0.88

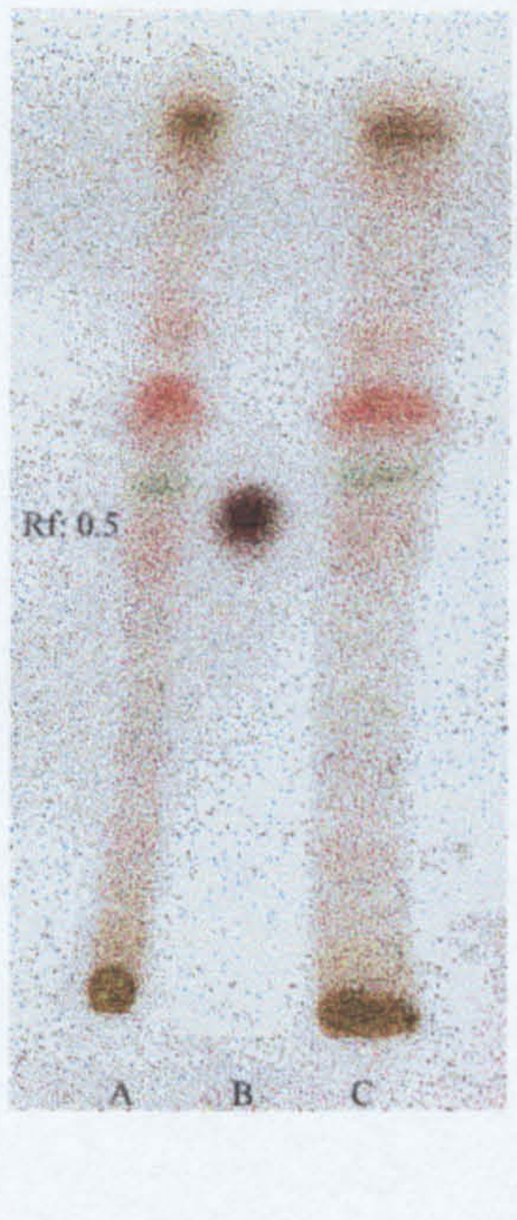


Figure 2.7. TLC profiles of *C. majalis* leaf EtOH extract (A, C) and cymarin (B). Chromatography conditions: stationary phase: silica gel ($60F_{254}$), mobile phase: less polar phase of DCM : MeOH : H_2O (5:6:4), spray reagent: acidic anisaldehyde.

Table 2.6. R_f values of zones from *C. majalis* leaf ethanolic extract (corresponding to Figure 2.8), examined in daylight (detection using acidic anisaldehyde, * examination under 366nm UV light).

Zone on TLC Plate	**R_f value**
Brown (Blue*)	0.01
Brown/Yellow	0.05
Blue*	0.15
Pink	0.33
Pink	0.57
Green (Orange*)	0.61
Deep Pink	0.68
Pink	0.73
Pink	0.83
Green (Orange*)	0.88

Figure 2.8. TLC profiles of *C. majalis* leaf EtOH extract (A, C) and cymarin (B). Chromatography conditions: stationary phase: silica gel ($60F_{254}$), mobile phase: less polar phase of $CHCl_3$: MeOH : H_2O (5:6:4), spray reagent: acidic anisaldehyde.

Table 2.7. R_f values of zones from *C. majalis* leaf ethanolic extract (corresponding to Figure 2.9), examined in daylight (detection using acidic anisaldehyde, * examination under 366nm UV light).

Zone on TLC Plate	**R_f value**
Yellow/Brown	0.07
Brown	0.12
Yellow/Brown	0.15
Green/Brown	0.25
Blue*	0.32
Pink	0.36
Green/Brown	0.47
Pink	0.64
Pink	0.84
Brown (Orange*)	0.91
Green (Orange*)	0.96

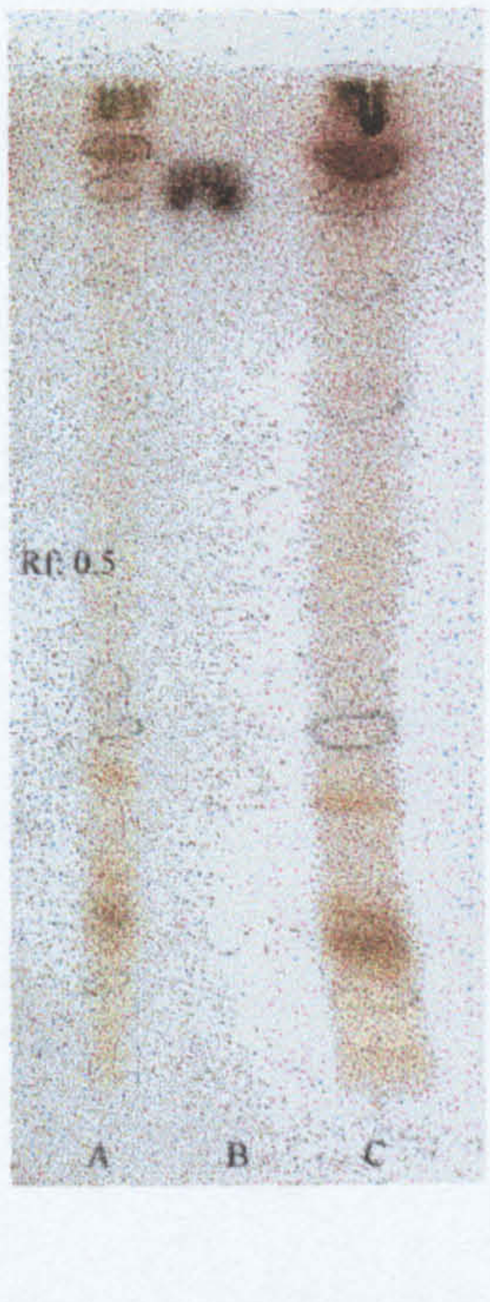


Figure 2.9. TLC profiles of *C. majalis* leaf EtOH extract (A, C) and cymarin (B). Chromatography conditions: stationary phase: silica gel ($60F_{254}$), mobile phase: less polar phase of $CHCl_3$: MeOH : ^nPrOH : H_2O (5:6:1:4), spray reagent: acidic anisaldehyde.

Table 2.8. R_f values of zones from *C. majalis* leaf ethanolic extract (corresponding to Figure 2.10), examined in daylight (detection using acidic anisaldehyde, * examination under 366nm UV light).

Zone on TLC Plate	**R_f value**
Yellow/Brown	0.10
Brown	0.16
Yellow/Brown	0.21
Green/Brown	0.33
Pink	0.41
Green/Brown	0.53
Pink	0.71
Pink	0.89
Brown (Orange*)	0.91
Green (Orange*)	0.95

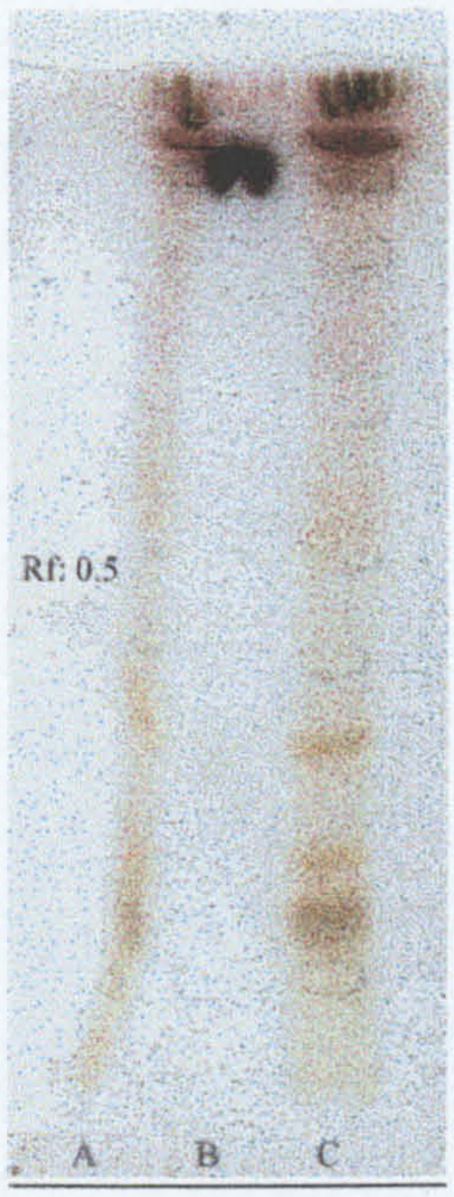


Figure 2.10. TLC profiles of *C. majalis* leaf EtOH extract (A, C) and cymarin (B). Chromatography conditions: stationary phase: silica gel ($60F_{254}$), mobile phase: less polar phase of $CHCl_3$: MeOH : ^nPrOH : H_2O (45:70:5:40), spray reagent: acidic anisaldehyde.

2.2.2.5 Preparative TLC: *C. majalis* Leaf Ethanolic Extract

After the plate was developed using the less polar phase of the solvent system: DCM : MeOH : H_2O (5:6:4), the plate was analysed under UV light (at 254nm and at 366nm) and a small section of the plate was sprayed with acidic anisaldehyde reagent (Figure 2.11); the plate was divided into 7 sections.

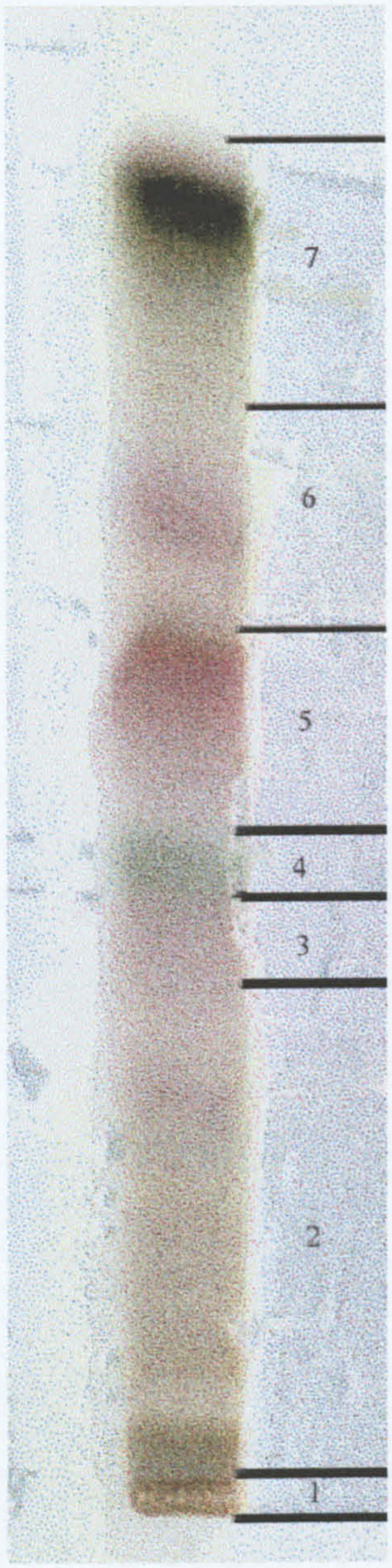


Figure 2.11. TLC profiles of *C. majalis* leaf EtOH extract, divided into 7 sections for preparative TLC. Chromatography conditions: stationary phase: silica gel ($60F_{254}$), mobile phase: less polar phase of DCM : MeOH : H_2O (5:6:4), spray reagent: acidic anisaldehyde.

Each of the 7 fractions, from each section removed from the plate, was analysed by application to silica gel ($60F_{254}$) plates, and developed using the solvent system $C_6H_5CH_3$: EtOAc : MeOH (5:6:4). The plate was examined under UV light (at 254nm and at 366nm) and in daylight after spraying with acidic anisaldehyde reagent (Figure 2.12 and Table 2.9). The results show that F2, F3 and F4 contain a similar zone (R_f value: 0.66). F3 and F4 showed similar antiChE activity (refer to Chapter 3, 3.2.4.5). This suggests that the same compound(s) present in these fractions may be ChE inhibitors. Three zones (R_f values: 0.37, 0.55, 0.66) were present in F3 and F4, the zones with R_f values of 0.37 and 0.55 being also present in F7. F3, F4 and F7 were the most active of all the preparative TLC fractions tested for antiChE activity (refer to Chapter 3, 3.2.4.5). Therefore, the compounds giving zones with the R_f values of 0.37 and 0.55 may be the active antiChE constituents. The compound(s) in the zones with the R_f values of 0.37 and 0.55 may contribute to antiChE activity by acting synergistically with other compounds, but they may not be the most active compound(s) of those present in the fractions (as presence in other fractions (either F5 or F6) did not confer high antiChE activity).

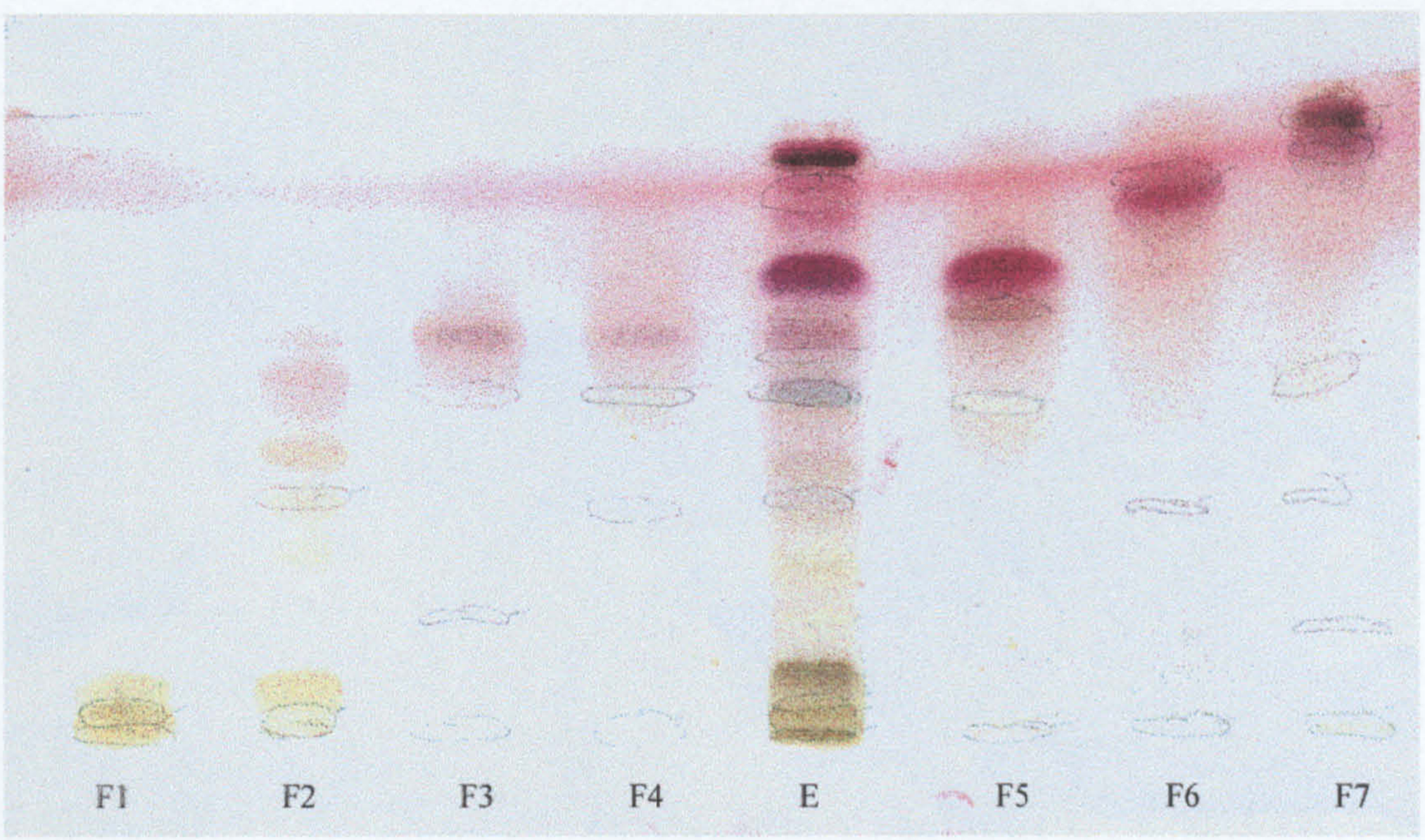


Figure 2.12. TLC profiles of preparative TLC fractions from *C. majalis* leaf EtOH extract. Chromatography conditions: stationary phase: silica gel ($60F_{254}$), mobile phase: $C_6H_5CH_3$: EtOAc : MeOH (5:6:4), spray reagent: acidic anisaldehyde. E = crude ethanolic extract of *C. majalis* leaf.

Table 2.9. Zones in 7 *C.majalis* leaf preparative TLC fractions; chromatography conditions: stationary phase: silica gel ($60F_{254}$), mobile phase: $C_6H_5CH_3$: EtOAc : MeOH (5:6:4) (Y = present; N = absent; * = detection using acidic anisaldehyde).

R_f value	**Day Light***	**UV Light 254nm**	**UV Light 366nm**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
0.01	Yellow	Y	Blue	Y	N	N	N	N	N	N
0.01	Yellow	Y	Green	N	Y	N	N	N	N	N
0.01	-	-	Grey	N	N	Y	N	N	N	N
0.01	-	-	Grey/ Orange	N	N	N	Y	N	N	N
0.01	Brown	-	Grey/ Brown	N	N	N	N	Y	Y	Y
0.05	Yellow	-	Blue/ Grey	Y	N	N	N	N	N	N
0.08	Yellow	-	-	Y	Y	N	N	N	N	N
0.20	-	-	Green	N	N	Y	N	N	N	Y
0.30	Yellow	-	-	N	Y	N	N	N	N	N
0.37*	-	Y	Orange	N	N	Y	Y	N	Y	Y
0.38	Yellow	-	Orange	N	Y	N	N	N	N	N
0.45	Yellow/ Pink	-	-	N	Y	N	N	N	N	N
0.55 **	Green	-	Orange	N	N	Y	Y	Y	N	Y
0.57	Pink	-	-	N	Y	N	N	N	N	N
0.66 ***	Pink	-	-	N	Y	Y	Y	N	N	N
0.69	Brown	Y	Orange	N	N	N	N	Y	N	N
0.76	Pink	-	-	N	N	N	N	Y	Y	Y
0.87	Pink	-	-	N	N	N	N	N	Y	N
0.90	-	-	Orange	N	N	N	N	N	Y	Y
0.94	Green	Y		N	N	N	N	N	N	Y
0.96	Pink	-	Orange	N	N	N	N	N	N	Y

An active fraction (F8a) from FCC (a) (refer to Chapter 3, 3.2.4.2) was compared with the compounds present in F3 and F4 from preparative TLC, to identify common compounds that may be responsible for antiChE activity. Three zones were found to be present in the 3 active fractions analysed, and also in the crude EtOH extract of *C. majalis* leaf (*, ** and ***, Figure 2.13 and Tables 2.9 and 2.10). It may be concluded that the compounds present in these 3 zones are responsible, either alone or in synergy, for the antiChE activity of the 3 active fractions and the crude extract. However, other compounds in each fraction may enhance the antiChE activity observed. Preparative TLC F7 also demonstrated notable antiChE activity (refer to Chapter 3, 3.2.4.5). This fraction contained 4 zones (R_f values: 0.20, 0.55, 0.94 and 0.96) not present in the much less active F6. One or more of these compounds in these zones may therefore have been responsible for the antiChE activity of this fraction.

Preparative TLC F3 and F4, F8a (from FCC (a)) and the crude EtOH extract of *C. majalis* leaf were also assessed for the presence of alkaloids, using Dragendorff's reagent for detection. No compounds were identified as alkaloids, since no orange zones were observed after spraying.

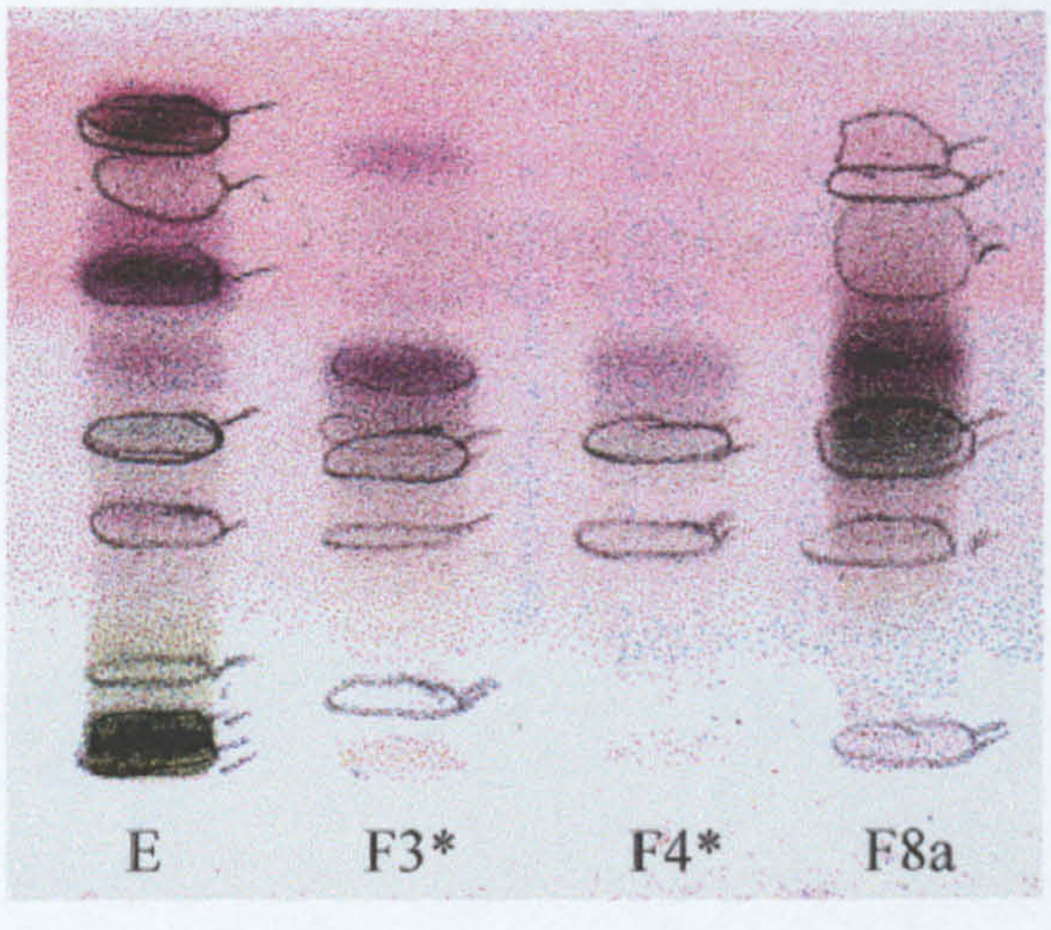


Figure 2.13. TLC profiles of preparative TLC fractions (F3* and F4*), and F8a (from FCC (a)), from *C. majalis* leaf EtOH extract. Chromatography conditions: stationary phase: silica gel ($60F_{254}$), mobile phase: $C_6H_5CH_3$: EtOAc : MeOH (5:6:4), spray reagent: acidic anisaldehyde.

Table 2.10. Zones present in F3 and F4 from preparative TLC separation, in F8a from FCC (a) separation, and in the crude ethanolic extract of *C. majalis* leaf (* = detection using acidic anisaldehyde).

Zone	R_f value	Daylight*	UV Light 254nm	UV Light 366nm
1*	0.29	-	Y	Orange
2**	0.43	Green	-	Orange
3***	0.52	Pink	-	-

2.2.3 LC-MS and HPLC Analysis of *C. majalis* Leaf Crude Ethanolic Extract and Preparative TLC Fractions

2.2.3.1 LC-MS Analysis of the Crude Ethanolic Extract of *C. majalis* Leaf

Analysis of the crude ethanolic extract of *C. majalis* leaf yielded information regarding the constituents present (refer to appendix, Figure A9). One peak at R_t 5.8min gave a pseudomolecular ion ($[M+H]^+$) at *m/z* 166 which gave a product ion spectrum after MS/MS analysis the same as phenylalanine. The peak at R_t 25.5min, showed a pseudomolecular ion ($[M+H]^+$) at *m/z* 1031, which corresponds to a glycoside since in MS/MS analysis it generated product ions showing successive losses of sugar moieties, leaving an aglycone product ion ($[A+H]^+$) at *m/z* 431. Loss of 146 (rmm) is likely to be due to loss of a rhamnosyl moiety, since in plants, rhamnosyl is the most common sugar of 164 (rmm) involved in glycosides (rarer sugars are fucose and quinovose). Similarly, the peak at R_t 26.2min gave a pseudomolecular ion ($[M+H]^+$) at *m/z* 1033, which gave a product ion spectrum also showing successive losses of sugar moieties after MS/MS analysis, with the corresponding aglycone ion ($[A+H]^+$) at *m/z* 433. The overlapping peaks at R_t 31.9 and R_t: 31.1 gave ions ($[M+H]^+$) at *m/z* 871 and *m/z* 869 respectively, which in MS/MS analysis, both showed losses of 3 hexosyl sugar moieties (rmm: 164) to give the aglycone ions ($[A+H]^+$) at *m/z* 433 and *m/z* 431 respectively.

The peak at R_t 36.8 gave an ion ($[M+H]^+$) at *m/z* 621, which in MS/MS analysis did not fragment, even when the collision energy was increased. The compound giving an ion ($[M+H]^+$) at *m/z* 391 was due to a contaminant (plasticiser) in the system, and

was therefore also evident in the chromatograms of the 7 preparative TLC fractions analysed. LC-MS analysis indicates that the crude EtOH extract of *C. majalis* leaf contains phenylalanine, glycosides and other unidentified compounds. Further investigation is required to establish if the glycosides present in the leaf EtOH extract are cardiac glycosides. Those glycosides (with ions at *m/z* 1031, 1033, 871 and 869) identified are not the cardiac glycosides convallatoxin (**50**) (rmm 550.7), reported to occur in *C. majalis* leaf, or cymarin (**48**) (rmm 548.7). Isolation and identification of the constituents present in the EtOH extract is required for their identification, but could not be carried out due to time limitations.

2.2.3.2 LC-MS and HPLC Analysis of Preparative TLC Fractions of *C. majalis* Leaf

Phenylalanine was present in both F1 and F2 (refer to appendix, Figures A10 and A11). Phenylalanine is a polar amino acid, so its presence in the more polar fractions would be expected.

The chromatogram for F2 (refer to appendix, Figure A11) showed two major peaks at R_t 21.9min and R_t 22.5min, each composed of two compounds, which gave psedomolecular ions ($[M+H]^+$) at *m/z* 922 and *m/z* 924 respectively. Analysis of the product ion spectra showed these to be glycosides, as MS/MS analysis showed successive losses of 3 hexosyl sugars (rmm 164) to yield the aglycone ions ($[A+H]^+$) at *m/z* 484 (for $[M+H]^+$ at *m/z* 922) and at *m/z* 486 (for $[M+H]^+$ at *m/z* 924). The peak at R_t 21.0min ($[M+H]^+$ at *m/z* 871 and $[A+H]^+$ at *m/z* 433) was also detected in F2. The presence of glycosidic compounds in a relatively polar fraction would be expected, as such compounds are generally polar.

The peaks at R_t 20.0min and R_t 20.8min were present in the chromatograms of both F3 and F4 (refer to appendix, Figures A12 and A14), which gave pseudomolecular ions ($[M+H]^+$) at *m/z* 277 and *m/z* 279 respectively. The ion at *m/z* 277 was also present in the chromatogram of F5 (refer to appendix, Figure A15), and the ion at *m/z* 279 was also evident in the chromatograms of F6 and F7 (refer to appendix, Figures A16 and F17). Both F3 and F4 inhibited AChE activity in the *in vitro* studies (refer to Chapter 3, 3.2.4.5), which suggests that compounds common to both fractions (e.g. ions at *m/z* 277 and *m/z* 279) may be AChE inhibitors. The ions at *m/z* 277 and at *m/z* 279 were also observed in the chromatograms of F6b and F7b (from FCC (b)) (refer to appendix, Figures A18 and A19). The presence of these compounds in the active

F6b and F7b suggests that they may also have contributed to the antiChE activity of these fractions *in vitro* (refer to Chapter 3, 3.2.4.3). The peak at R_t 20.0min, which gave the pseudomolecular ion ($[M+H]^+$) at *m/z* 277, gave fragment ions at *m/z* 135 and *m/z* 121 after MS/MS analysis, often seen in the MS/MS of flavonoids.

Therefore, F3 was also analysed using HPLC and photodiode array detection, to investigate the presence of flavonoids. Two of the compounds identified had UV spectral maxima at 240nm (refer to appendix, Figure A13); however the UV spectra were not characteristic of any known flavonoids, suggesting the absence of flavonoids in F3. In F7, the peak at R_t 26.3 also gave the ion ($[M+H]^+$) at *m/z* 621 (refer to appendix, Figure A17), which in MS/MS analysis did not fragment.

LC-MS analysis has provided information suggesting that compounds yielding ions at *m/z* 277 or *m/z* 279 may be responsible for antiChE activity, and that these compounds do not appear to be flavonoids. However, it cannot be excluded that other components present in the crude EtOH extract or in the fractions are responsible, perhaps in synergy, for antiChE activity. In conclusion, it is apparent that further fractionation and isolation is required to identify the compounds responsible for antiChE activity *in vitro*.

2.2.4 Analysis of *C. majalis* Leaf Extracts Obtained by Soxhlet Extraction

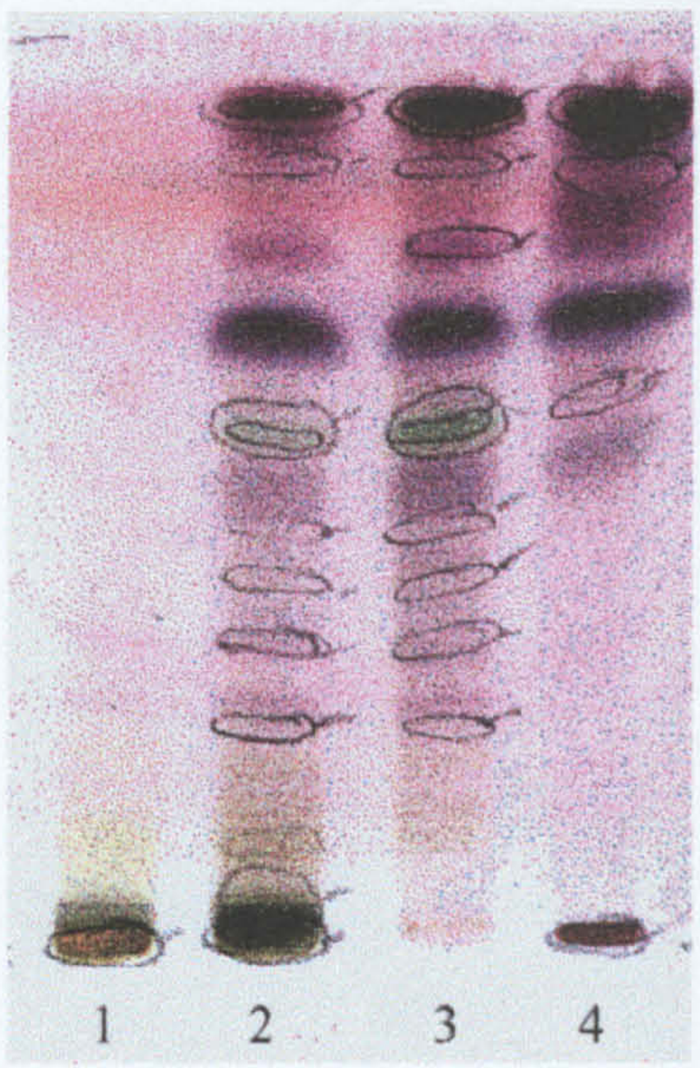


Figure 2.14. TLC profiles of *C. majalis* leaf extracts (1: H_2O, 2: EtOH, 3: DCM, 4: C_6H_{14} extracts). Chromatography conditions: stationary phase: silica gel ($60F_{254}$), mobile phase: less polar phase of DCM : MeOH : H_2O (5:6:4), spray reagent: acidic anisaldehyde.

TLC analysis showed the EtOH and DCM extracts to contain a similar chemical profile. As expected, some the more polar compounds (e.g. glycosides) present in the EtOH extract were absent in the DCM and C_6H_{14} extracts (Figure 2.14). Similarly, compounds present in the EtOH and DCM fractions were absent in the less polar C_6H_{14} extract (Figure 2.14).

2.2.5 Comparison of the Ethanolic Extract of *C. majalis* Leaf with Chlorophyll

Comparison of the EtOH extract of *C. majalis* leaf with chlorophyll, using the solvent system DCM : MeOH (9:1), showed two corresponding zones; one violet zone visible in daylight (R_f: 0.52) after spraying with acidic anisaldehyde, and one green zone, visible in UV light (366nm) (R_f: 0.74) (Figures 2.15 and 2.16).

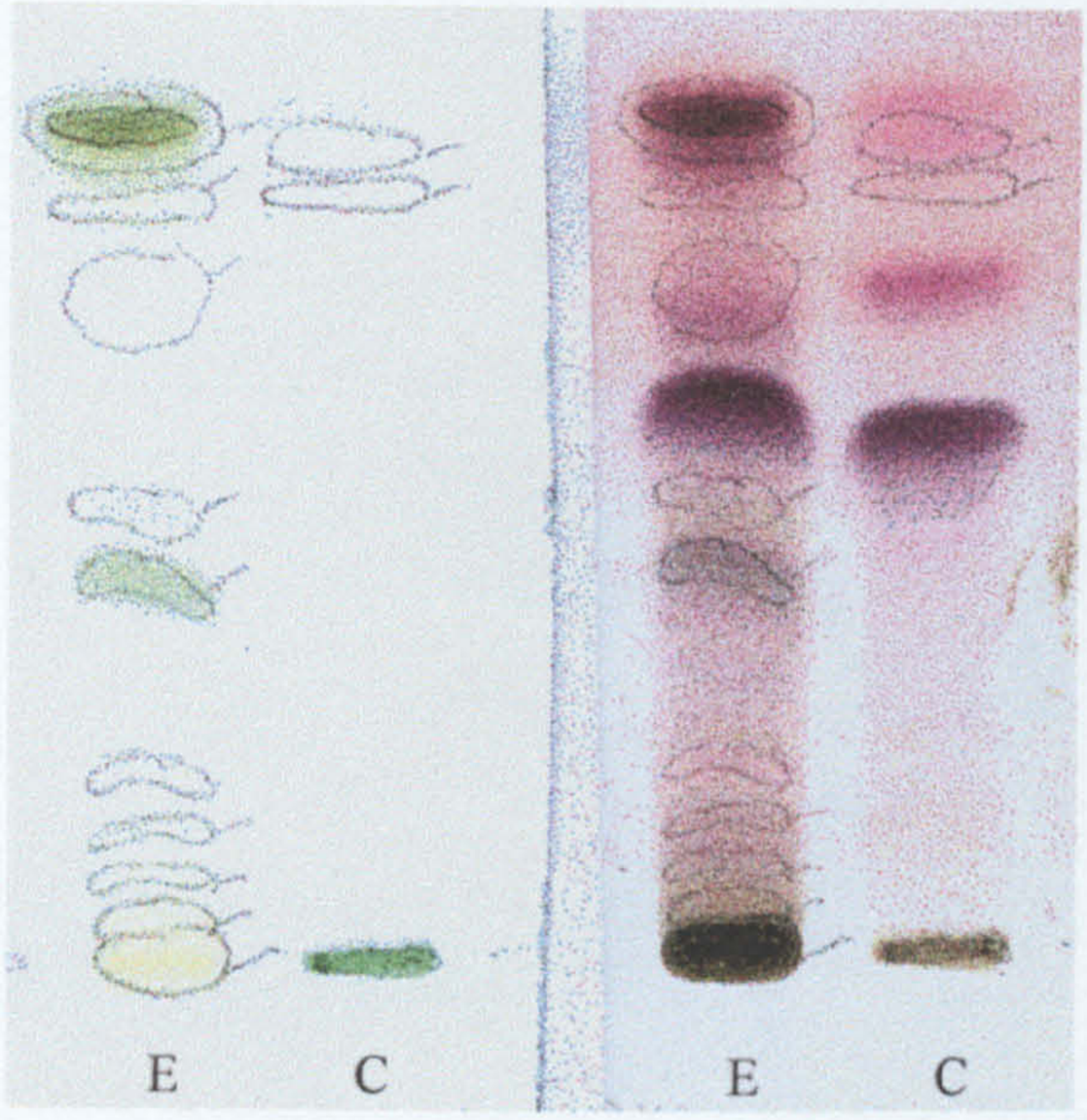


Figure 2.15 **Figure 2.16**

Figures 2.15. and 2.16. TLC profiles of *C. majalis* leaf EtOH extract (E) and chlorophyll (C). Chromatography conditions: stationary phase: silica gel ($60F_{254}$), mobile phase: DCM : MeOH (9:1), spray reagent: acidic anisaldehyde. **Figure 2.15.** Chromatogram before spraying. **Figure 2.16.** Chromatogram after spraying.

Using the solvent system DCM : MeOH (1:1) the violet zone was again evident in daylight after spraying with acidic anisaldehyde (R_f: 0.54), as were other violet zones (R_f: 0.65 and R_f: 0.86). In UV light (366nm) the green zone was again visible (R_f: 0.77). In daylight before spraying, both plates did not show any corresponding zones for the extract and chlorophyll, however in UV light (254nm) a dark zone was visible at the baseline of each plate for both the extract and chlorophyll. The ethanolic extraction of *C. majalis* leaf would be expected to yield many components including chlorophyll, other pigments and lipids, as well as the cardenolide compounds. The subsequent separation of compounds from this extract (using FCC, DCCC and preparative TLC) enables all the compounds to be assessed for antiChE activity; consequently any effects of compounds such as chlorophyll on AChE activity can be taken into consideration.

2.2.6 Analysis of Herbal Extracts for the Presence of GABA

Analysis for GABA (**16**) was initially investigated using cellulose plates for TLC analysis. Silica gel plates are also appropriate for analysis (Stahl, 1969) and were therefore used for further analysis. GABA (**16**) (2μg) was applied to the TLC plate for reference; with the solvent system butan-1-ol : H_2O : CH_3COOH (60:25:15). The R_f values for GABA (**16**) using separate TLC plates were 0.23, 0.28 (Figure 2.17), 0.24, 0.26 and 0.25; R_f values may have differed due to different elution temperatures. All extracts with a zone detected with ninhydrin, with a R_f value corresponding to that of GABA (**16**), were regarded as being positive for GABA (**16**) (for example, Figure 2.17).

GABA (**16**) was detected in all extracts, except *Melissa officinalis* leaf aqueous extract, *Melissa officinalis* leaf ethanolic extract, *Rosmarinus officinalis* dried leaf ethanolic extract, *Rosmarinus officinalis* fresh leaf ethanolic extract, *Ziziphus jujuba* var. *spinosa* seed aqueous extract and *Ziziphus jujuba* var. *spinosa* seed ethanolic extract.

GABA (**16**) is reported to be regularly present in plants (Harborne and Baxter, 1993), therefore the identification of GABA (**16**) in 22 of the 28 plant extracts analysed is not unexpected. Detection with ninhydrin also showed amino acids other than GABA to occur in the plant extracts (Figure 2.17). The presence of amino acids in plants may be important for nitrogen storage.

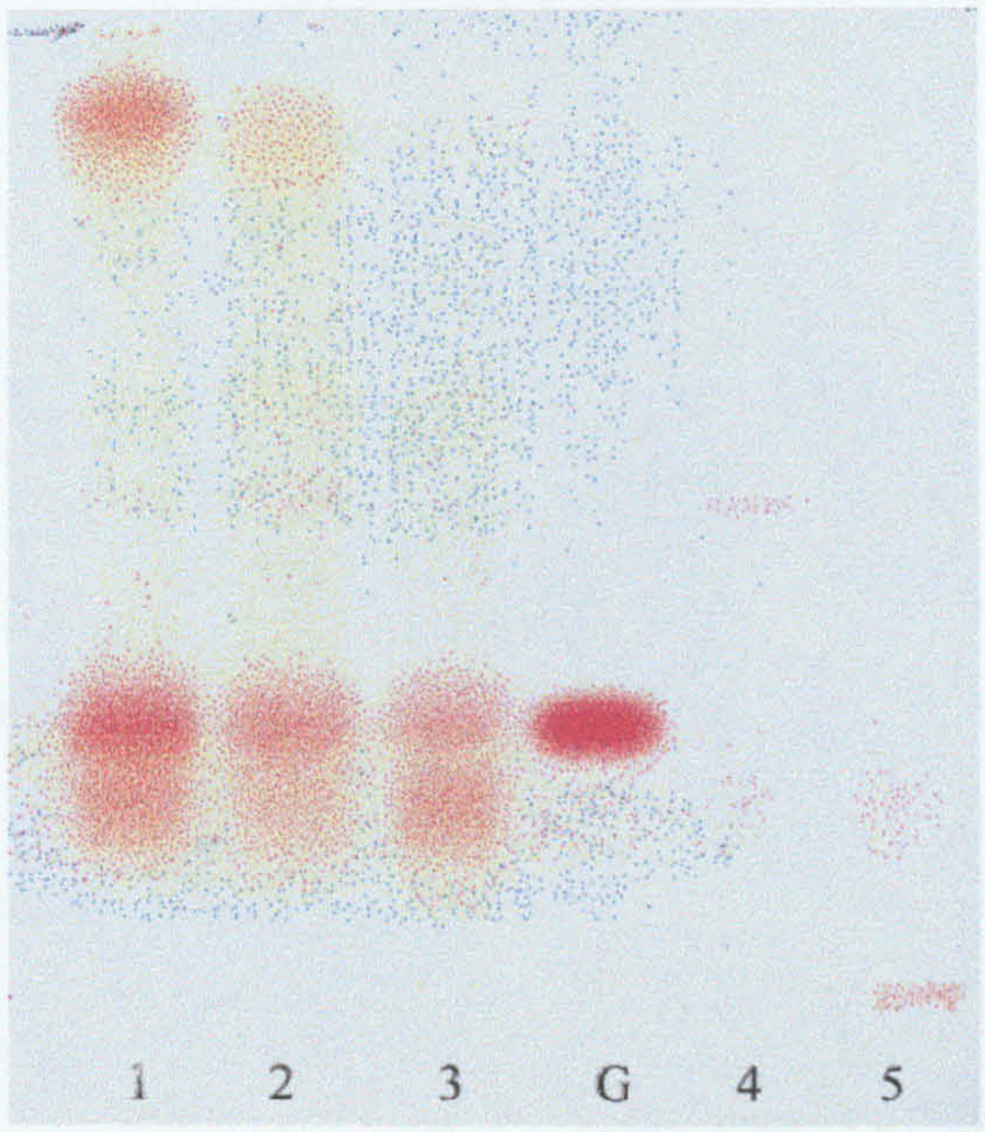


Figure 2.17. TLC profiles of some plant extracts (1: *Salvia miltiorrhiza* root EtOH extract, 2: *Salvia miltiorrhiza* root H_2O extract (batch 1), 3: *Salvia miltiorrhiza* root H_2O extract (batch 2), 4: *Ziziphus jujuba* var. *spinosa* seed EtOH extract, 5: *Ziziphus jujuba* var. *spinosa* seed H_2O extract) and reference solution: GABA (G). Chromatography conditions: stationary phase: silica gel ($60F_{254}$), mobile phase: butan-1-ol : H_2O : CH_3COOH (60:25:15), spray reagent: ninhydrin.

2.2.7 Gas Chromatography-Mass Spectroscopy

2.2.7.1 Analysis of *Melissa officinalis* Essential Oil Using GC-MS

Melissa officinalis essential oil (Fragrant Earth Co.) was obtained from the leaves of *M. officinalis* by the process of steam distillation. During this process, steam is passed through the herb, which results in the volatile essential oil from the herb condensing in a collection chamber. This process is a reproducible and rapid method for obtaining large quantities of volatile oils. A disadvantage of this process is that some volatile oil constituents may undergo chemical changes due to exposure to water and due to the high temperature during the distillation process. Compounds may undergo rearrangement, isomerisation or oxidation. Some compounds may hydrolyse (e.g. the esters), and others may decompose at the high temperatures used.

Consequently, the chemical composition of the oil collected may differ from that of the oil in the original plant.

M. officinalis phytol extract is obtained not by steam distillation, but by a process of 'phytonics'. This process involves washing the plant material with a low boiling solvent (e.g. 1, 1, 1, 2-tetrafluoroethane, which has a boiling point of -26.2°C). The washings are transferred to a vessel where the solvent is allowed to evaporate at room temperature since its boiling point is less than 0°C. The evaporated solvent is re-condensed, and the resulting liquid used for further extraction from the herb. This process is of advantage over the steam distillation method, as none of the volatile components are lost by decomposition or hydrolysation, and is a preferred method over standard solvent extraction, which often involves higher temperatures to evaporate the solvent.

Both the *M. officinalis* phytol extract and the crude phytol extract (which was not refined by removal of non-volatile oil components) were analysed for their chemical composition using GC-MS (refer to Table 2.11 and appendix, Figures A20 and A21). GC is a suitable analytical method as the essential oils are volatile, and results can be obtained within a relatively short time. The chromatograms for the three essential oils analysed show that they consist of a complex mixture of compounds which have differences in polarity and volatility. Some components may have low thermal stability. This may be a problem, as the formation of artifacts may arise, which will be detected and may be regarded as an original component of the essential oil.

The chemical profiles of *M. officinalis* phytol and the crude phytol extracts were compared with the *M. officinalis* essential oil obtained by steam distillation (refer to Tables 2.11 and 2.12 and appendix, Figures A20, A21 and A22). The results show that the chemical composition of the oils analysed differs. This may be due to the different methods of extraction, differences arising from the storage conditions or differences between the *M. officinalis* plants due to environmental factors (e.g. cultivation conditions such as temperature, climate, soil, duration of daylight, or the time of year harvested). Differences in the chemical profile between the oils may also occur as a result of different subspecies being used for extraction of the oil. For example, the oil from the *altissima* subspecies has been reported to differ from that of the subspecies *inodora* (Dawson *et al.*, 1988; Şarer and Kökdil, 1991). Genetic variability may also be a contributing factor to the variation in essential oil composition (Adzet, *et al.*, 1992).

Table 2.11. Percentage composition of *Melissa officinalis* phytol and crude phytol extracts (Clwydian Fragrant Oil) determined by GC-MS analysis.

No	Compound	Retention time (s)	*Melissa* Phytol % comp. TIC	*Melissa* Phytol % comp. FID*	*Melissa* Phytol % comp. FID	*Melissa* Phytol (Crude) % comp. TIC	*Melissa* Phytol (Crude) % comp. FID*	*Melissa* Phytol (Crude) % comp. FID
	α-thujene	212	0.09	0.18	0.23	0.13	0.32	0.53
1	α-pinene	222	0.05	0.12	0.16	0.08	0.78	1.30
2	1-octen-3-ol	292	0.18	0.29	0.38	0.12	0.59	0.98
3	6-methyl-5-hepten-2-one	-	-	-	-	-	-	-
4	limonene	-	-	-	-	-	-	-
	cis-β-ocimene	380	0.12	0.22	0.28	0.12	0.32	0.53
5	*trans*-β-ocimene	400	1.73	1.75	2.27	1.87	1.43	2.38
6	linalool	520	0.23	0.31	0.40	0.20	0.29	0.48
7	*cis*-rose oxide	540	0.05	0.16	0.21	0.05	0.10	0.17
8	unidentified	-	-					
9	unidentified	-	-					
10	citronellal	648	4.65	3.80	4.92	4.85	2.83	4.71
11	unidentified	672	0.06	0.12	0.16	0.06	0.07	0.12
12	unidentified	722	0.08	0.17	0.22	0.10	0.19	0.32
13	dodecane	-	-					
14	nerol	832	5.72	1.52	1.97	7.73	**	**
15	citronellol	840	1.19	**	**	**	**	**
16	neral	879	15.24	13.5	17.48	15.50	9.66	16.07
17	geraniol	906	13.62	6.26	8.11	12.59	2.1	3.49
18	methyl citronellate	-	-					
19	geranial	963	22.69	22.15	28.69	22.59	20.79	34.59
20	methyl geranate	1084	0.32	1.63	2.11	0.28	0.32	0.53
	eugenol	1178	0.42	0.20	0.26	0.30	0.40	0.67
21	α-copaene	1195	0.65	0.52	0.67	0.62	1.37	2.28
	unknown	1210	2.15	2.11	2.73	3.09	0.66	1.10
22	β-burbonene	-	-					
23	β-cubebene		-					
	geranyl acetate	1231	2.35	1.57	2.03	2.25	1.22	2.03
24	β-elemene	-	-					
25	tetradecane	-	-					
26	*trans*-caryophyllene	1311	9.20	7.21	9.34	9.25	5.46	9.08
27	unidentified	-	-					
28	α-humulene	1404	1.05	0.53	0.69	0.95	0.41	0.68
29	γ-muurolene	1458	0.11	0.23	0.30	0.12	0.17	0.28
30	germacrene-D	1474	9.78	7.00	9.07	9.30	5.26	8.75
31	unidentified	1504	1.61	1.07	1.39	1.42	0.86	1.43
32	α-muurolene	1520	0.13	0.12	0.16	0.15	0.11	0.18
33	(E, E)-α-farnesene	1538	0.64	0.52	0.67	0.57	0.41	0.68
34	γ-cadinene	1556	0.34	0.31	0.40	0.34	0.29	0.48
35	δ-cadinene	1567	0.78	0.64	0.83	0.96	0.62	1.03
36	germacrene-D-4-ol	1721	3.93	1.72	2.23	3.40	1.76	2.93
37	caryophyllene oxide	1732	0.28	0.56	0.73	0.19	0.50	0.83
38	hexadecane	-	-					
39	epi-α-cadinol	1884	0.10	0.17	0.22	0.12	0.18	0.30
30	δ-cadinol	1891	0.12	0.17	0.22	0.17	0.23	0.38
41	unidentified	1918	0.34	0.38	0.49	0.53	0.41	0.68
42	unidentified	-	-					
	TOTAL	-	**100**	**77.21**	**100**	**100**	**66.11**	**100**

* based on all peaks ** % composition unidentified due to inadequate resolution of peaks

Table 2.12. Percentage composition of *Melissa officinalis* essential oil (Fragrant Earth) determined by GC-MS analysis.

No	Compound	Retention time (s)	% comp. TIC	% comp. FID	% comp. FID*
1	α-pinene	227	0.08	0.43	0.34
2	1-octen-3-ol	297	0.34	0.29	0.23
3	6-methyl-5-hepten-2-one	308	1.45	2.11	1.69
4	limonene	375	0.13	0.44	0.35
5	*trans*-β-ocimene	406	0.74	1.49	1.19
6	linalool	527	0.71	1.46	1.17
7	*cis*-rose oxide	547	0.22	1.05	0.84
8	unidentified	625	0.17	1.25	1.00
9	unidentified	651	0.30	0.48	0.38
10	citronellal	656	1.42	2.00	1.60
11	unidentified	681	0.13	1.05	0.84
12	unidentified	728	0.50	1.54	1.23
13	dodecane	746	0.17	0.36	0.29
14	nerol	839	0.75	1.45	1.16
15	citronellol	847	0.41	0.88	0.70
16	neral	890	12.57	11.13	8.90
17	geraniol	911	1.23	1.11	0.89
18	methyl citronellate	920	0.56	0.50	0.40
19	geranial	974	16.57	14.73	11.78
20	methyl geranate	1094	0.41	2.06	1.65
21	α-copaene	1207	1.34	1.76	1.41
22	β-burbonene	1227	0.40	2.44	1.95
23	β-cubebene	1244	2.00	0.69	0.55
24	β-elemene	1244	2.31	0.80	0.64
25	tetradecane	1273	0.28	0.39	0.31
26	*trans*-caryophyllene	1330	28.39	23.66	18.92
27	unidentified	1351	0.09	0.70	0.56
28	α-humulene	1418	2.60	1.78	1.42
29	γ-muurolene	1472	0.23	0.54	0.43
30	germacrene-D	1490	12.66	9.76	7.80
31	unidentified	1516	0.63	0.89	0.71
32	α-muurolene	1533	0.60	0.71	0.57
33	(E, E)-α-farnesene	1551	0.67	0.69	0.55
34	γ-cadinene	1570	0.44	0.61	0.49
35	δ-cadinene	1582	2.64	2.13	1.70
36	germacrene-D-4-ol	1734	0.27	1.75	1.40
37	caryophyllene oxide	1747	2.56	1.75	1.40
38	hexadecane	1772	0.58	0.18	0.14
39	epi-α-cadinol	1898	0.42	0.45	0.36
30	δ-cadinol	1904	0.53	0.73	0.58
41	unidentified	1932	1.23	1.19	0.95
42	unidentified	2335	0.30	0.60	0.48
	TOTAL	-	**100**	**100**	**79.95**

* based on all peaks

Adulteration can be a problem with *M. officinalis* oil, due to the low yield of the oil from the plant and the subsequent production cost. *M. officinalis* oil has been reported to be adulterated with lemongrass oil and citronella oil (Guenther, 1949). Compounds that were detected in *M. officinalis* phytol extract, but not in the steam distilled *M. officinalis* oil, were ocimene (**76**), eugenol (**84**) and gernanyl acetate (Tables 2.11 and 2.12). Compounds detected in the steam distilled *M. officinalis* oil, but not in *M. officinalis* phytol extract, were 6-methyl-5-hepten-2-one (**85**), limonene (**73**), dodecane, methyl citronellate, β-burbonene, β-cubebene (**80**), β-elemene, tetradecane and hexadecane (Tables 2.11 and 2.12).

Typically, the compounds present in the essential oils analysed are largely monoterpenoids (e.g. geranial (**70**) and neral (**71**)). The phenylpropanoid eugenol (**84**) was detected in the *M. officinalis* phytol extract. Phenylpropanoids are another group of constituents often present in essential oils but it is the terpenoids which predominate in the *M. officinalis* oils. It has been reported that *M. officinalis* oil contains >60% monoterpenes and >35% sesquiterpenes (Bisset, 1994). Citral (geranial (**70**) and neral (**71**)) comprised 35.7% of *M. officinalis* phytol extract, 30.5% of *M. officinalis* phytol extract (crude) and 20.7% of *M. officinalis* oil. Citral has been reported to account for 10% - 30% of *M. officinalis* essential oil (Bisset, 1994). The higher citral content in the phytol extracts may be due to the less destructive extraction procedure, and/or storage conditions of the oil (although other factors may also be responsible, as discussed previously). Neral (**71**) and geranial (**70**) occurred in the ratio 3:5 in the *M. officinalis* phytol extract, 1:2 in the *M. officinalis* phytol extract (crude) and 3:4 in *M. officinalis* oil. A ratio of 3:4-5 (geranial (**70**) : neral (**71**)) (Bisset, 1994), and of 4:5 (neral (**71**) : geranial (**70**)) (Şarer and Kökdil, 1991) has previously been reported to occur in *M. officinalis* essential oil.

The essential oils were analysed using two methods of detection: FID and MS, giving the TIC. MS determines peak area by counting ions. Therefore, if a compound (A) forms more molecular ions than another compound (B), then the peak area of A will be greater than that of B, even if they are present in equimolar amounts. FID gives results for peak area based on carbon number, and could therefore be considered a more reliable method for assessment of essential oil composition. The essential oils listed in the European Pharmacopoeia (2001) and the British Pharmacopoeia (1998) are assessed using GC with FID. The United States Pharmacopoeia (2000) includes a monograph for eucalyptol, obtained from oil of eucalyptus, which is also analysed by

using GC with FID. It could be assumed that these methods of analysis would have been tested for reliability and reproducibility prior to inclusion in the Pharmacopoeias. Therefore, it may be concluded that FID is the more reliable method of assessment.

2.2.7.2 Analysis of Commercially Obtained Oil Constituents Using GC-MS

Commercially obtained essential oil constituents were analysed to determine their purity, and to confirm their identification in the *M. officinalis* oils analysed. Citral is a mixture of two isomeric aldehydes; geranial (**70**) (the *trans*-isomer) and neral (**71**) (the *cis*-isomer). Therefore it is expected that both isomers would be detected in the GC-MS analysis of commercial citral. One source of citral (Aldrich) was 98.7% pure, containing a greater proportion of geranial (**70**) than neral (**71**) (refer to Table 2.13 and appendix, Figure A23).

Table 2.13. Percentage composition of citral (Aldrich) determined by GC-MS analysis.

Compound	**Retention time (s)**	**% Comp. TIC**
alkane	316	0.08
alkane (C12)	748	0.25
neral	887	38.88
geranial	970	59.78
unidentified	978	0.39
unidentified	1198	0.05
unidentified	1249	0.13
alkane (C14)	1272	0.08
alkane (C14)	1274	0.13
alkane (C16)	1778	0.13
alkane (C16)	1782	0.09
TOTAL	-	**100**

Geranial (**70**) was also the major isomer in another source of citral (Lancaster), which was 99.2% pure (refer to Table 2.14 and appendix, Figure A24). Other components were detected in these commercially obtained oils, including alkanes and unidentified

compounds. These may be degradation products of citral that may have occurred during storage, as exposure to light, O_2 and H_2O may have altered the chemical composition of the oils. Extraction and isolation procedures, the original source of citral, storage conditions and age of each oil may all have influenced the ratio of the isomers and this may explain the differences observed between sources of citral.

Table 2.14. Percentage composition of citral (Lancaster) determined by GC-MS analysis.

Compound	**Retention time (s)**	**% Comp. TIC**
alkane (C12)	739	0.15
neral	891	38.92
unidentified	937	0.11
geranial	974	60.25
unidentified	983	0.09
unidentified	1165	0.22
alkane (C14)	1276	0.09
alkane (C16)	1777	0.17
TOTAL	**-**	**100**

Linalool (**74**) was found to be 95.7% pure (refer to Table 2.15 and appendix, Figure A27) but also contained the oxidised derivatives of linalool (**74**) (*cis*- and *trans*-linalool oxide), dihydro-linalool, 1, 8-cineole (**56**), an alkane and an unidentified component, also detected using GC-MS.

The commercially obtained eugenol (**84**) was found to be 99.7% pure (refer to appendix, Figure A25) and the commercially obtained geraniol (**72**) was found to be 98.2% pure (refer to Table 2.16 and appendix, Figure A26). The commercially obtained nerol (**75**) was found to be 95% pure, the other oil constituents being identified as geranial (**70**) (1.8%), geraniol (**72**) (1.4%) and neral (**71**) (1.8%) (refer to appendix, Figure A28).

Table 2.15. Percentage composition of linalool (Aldrich) determined by GC-MS analysis.

Compound	**Retention time (s)**	**% Comp. TIC**
1, 8-cineole	388	0.10
cis-linalool oxide	465	0.88
trans-linalool oxide	501	0.89
linalool	536	95.66
dihydro-linalool	613	1.72
unidentified	765	0.63
alkane	1251	0.13
TOTAL	-	**100**

Table 2.16. Percentage composition of geraniol (Sigma) determined by GC-MS analysis.

Compound	**Retention time (s)**	**% Comp. TIC**
nerol	1082	1.0
neral	1109	0.2
geraniol	1159	98.2
geranial	1176	0.6
TOTAL	-	**100**

It may therefore be concluded that the ≥95% composition of each of the commercially obtained constituents, indicates that their activities in the bioassays are due to the major component of each oil, and the identification of the minor components of each oil enables any contribution to pharmacological activity of such components to be accounted for. Each of the major constituents citral, geraniol (**72**) and nerol (**75**) contain their chemically related monoterpenes (e.g. commercially obtained geraniol (**72**) also contains geranial (**70**), nerol (**75**) and neral (**71**) (Table 2.16)), therefore comparison of each monoterpene in the bioassays allows the significance of each of the other monoterpenes in observed pharmacological activities to be considered.

2.2.7.3 Assessment of Metabolism of Compounds by Yeast

The results from this investigation show that the peak areas of the compound under analysis decreased as the time at which the sample was collected from the assay was increased (refer to Chapter 4, Tables 4.3 - 4.5 and 4.8). This suggests that the concentration in the assay decreased with time (refer to Chapter 4, 4.7.2 and 4.10.2 for results and discussion).

CHAPTER 3

Assessment of Anticholinesterase Activity of Plant Extracts and Essential Oils

Cholinesterases are enzyme that hydrolyses esters of choline. Various methods have been described for the assessment of acetylcholinesterase (AChE) activity, including electrometric, titrimetric, and gasometric methods (Wilson and Henderson, 1992). Other techniques include measurement of the pH change which occurs upon ACh (**1**) hydrolysis (Hestrin, 1949), determination of [^{3}H]-ACh hydrolysis (Johnson and Russell, 1975), and measurement of the yellow 5-thio-2-nitrobenzoic acid anion which is formed by the reaction between the thiol group, released following hydrolysis of the substrate acetylthiocholine (ATCh), with 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB) (Ellman *et al.*, 1961). The latter photometric method is a sensitive and reproducible technique for assessment of AChE activity. Since erythrocyte AChE is reported to have similar properties to brain AChE (Sørensen *et al.*, 1982), the effect of the various plant extracts and essential oils on inhibition of human erythrocyte AChE activity was investigated to determine potential AChE inhibitors relevant to AD management. Extracts and essential oils were diluted in either H_2O, or in EtOH (<1%) at a concentration that did not interfere with AChE activity. Inhibition of erythrocyte AChE activity occurs with increasing EtOH concentration but effects on AChE activity are negligible at concentrations <2% (Haboubi and Thurnham, 1986; Ryan and Byrne, 1988).

3.1 AChE Inhibition Assay Methods

3.1.1 Materials

ATCh iodide, DTNB, human erythrocyte AChE and physostigmine (eserine hemisulfate salt) were obtained from Sigma, Fancy Road, Poole, Dorset, England. Na_2HPO_4 and NaH_2PO_4 were obtained from Fluka Chemicals, The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT, England. $NaHCO_3$ was obtained from

BDH Supplies, Poole, England. Plant material, essential oils, pure compounds (convallatoxin, cymarin and hyperoside) and chlorophyll were obtained as described previously (Chapter 2, 2.1.1.1); *Melissa officinalis* essential oil (organic) was obtained from Fragrant Earth, Taunton, England.

3.1.2 Preparation of AChE Assay Solutions

3.1.2.1 Preparation of Buffer Solutions

Sodium phosphate buffer solutions were prepared as follows:

pH	Na_2HPO_4 (0.2M)	H_2O	NaH_2PO_4 (0.2M)
7	47ml	50ml	1-3ml until pH 7
8	47ml	50ml	1-3ml until pH 8

Buffer solutions were stored at 4°C, and the pH of each solution confirmed prior to each assay using a 3305 Jenway pH meter.

3.1.2.2 Preparation of Assay Solutions

AChE was dissolved in phosphate buffer (pH 8) at a concentration of 0.26 units/ml (1 unit AChE hydrolyses 1.0 μmole.min^{-1} of ATCh at 37°C); ATCh was dissolved in distilled H_2O (0.6mM final assay concentration); DTNB was dissolved in phosphate buffer (pH 7) with 40mg $NaHCO_3$ per 5ml (0.5mM DTNB final assay concentration); physostigmine hemisulfate salt was dissolved in distilled H_2O (0.1mM final assay concentration). All assay solutions were freshly prepared for each assay, and were kept on ice (4°C) away from light.

Plant extracts, essential oils and compounds were diluted in EtOH prior to testing in the assay, and were stored at 4°C.

3.1.3 Investigation to Determine the Effect of Erythrocyte AChE Concentration on Breakdown of ATCh

A concentration range (0.76 units.L^{-1} - 9.81 units.L^{-1}) of human erythrocyte AChE (1µl equivalent to $2.6x10^{-4}$ units AChE) was incubated at 4°C in 2ml sodium phosphate buffer (pH 8) with 20µl of DTNB solution (triplicate readings repeated three times; n=3). After vortex mixing, the reaction was initiated by addition of 20µl of the solution containing the substrate ATCh, and vortex mixed again. After 20min at 37°C the reaction was terminated by addition of 20µl physostigmine hemisulfate salt solution to each tube at 4°C then the solutions were vortex mixed. The optical density was measured spectrophotometrically at 412nm, using a Shimadzu UV-VIS Scanning Spectrophotometer (CPS-260).

3.1.4 Investigation to Assess Effect of Plant Extracts, Essential Oils and Known Plant Constituents on Erythrocyte AChE Activity

Enzyme activity was measured in sodium phosphate buffer (pH 8). 40µl of enzyme solution (0.26 units/ml) was pre-incubated in 2ml phosphate buffer (pH 8) and 20µl of the test substance diluted in EtOH (or H_2O), at 4°C for 30min. Duplicate tubes were also treated this way in the presence of 20µl physostigmine hemisulfate salt solution to allow interference of the test substance in the assay to be assessed, and to control for any hydrolysis of ATCh not due to AChE activity. Controls (in the presence and absence of the AChE inhibitor physostigmine (**5**)) were pre-incubated with 20µl EtOH (or H_2O) instead of the test substance. To further control for the auto hydrolysis of ATCh in the assay, tubes were set up (2ml buffer, 20µl EtOH or 20µl H_2O) in the absence of AChE. All solutions were vortex mixed prior to pre-incubation at 4°C for 30min.

Following pre-incubation, 20µl aliquots of DTNB solution were added to each tube. After vortex mixing, 20µl aliquots of ATCh solution were added to each tube and solutions were vortex mixed prior to incubation at 37°C for 20min. Addition of 20µl physostigmine hemisulfate salt solution at 4°C terminated the reaction and solutions were vortex mixed. The optical density was measured at 412nm, using a Shimadzu UV-VIS Scanning Spectrophotometer (CPS-260).

3.1.5 Data Analysis

The enzyme activity was calculated as the difference between the absorbance of the initially uninhibited tubes, minus initially inhibited tubes, as follows:

Percentage AChE inhibition is calculated by:

$$\frac{\Delta_A - \Delta_B}{\Delta_A} \times 100 \qquad \Delta_A = A_C - B_C \qquad \Delta_B = A_P - B_P$$

B_C	B_P	A_C	A_P
ATCh	ATCh	ATCh	ATCh
DTNB	DTNB	DTNB	DTNB
EtOH / H_2O	Extract	EtOH / H_2O	Extract
AChE	AChE	AChE	AChE
Physostigmine	Physostigmine		

Significant activity against AChE, was regarded as >20% inhibition of the enzyme. Data are presented as the mean (n=3-6 ± SD). Concentration dependent data were analysed using one-way ANOVA to determine the significance of the difference. Significance was regarded as $p<0.05$.

3.2 Results and Discussion

3.2.1 Effect of Erythrocyte AChE Concentration on Breakdown of ATCh

Several successive experiments were performed to establish the effect of [AChE] on ATCh hydrolysis, and to determine a suitable concentration of AChE for screening plant extracts. Initial experiments showed that increase of AChE concentration

increases ATCh hydrolysis (p<0.05) (Figure 3.1). A concentration of 19.1µg/ml (4.97 units.L^{-1}) AChE was selected to screen plant extracts.

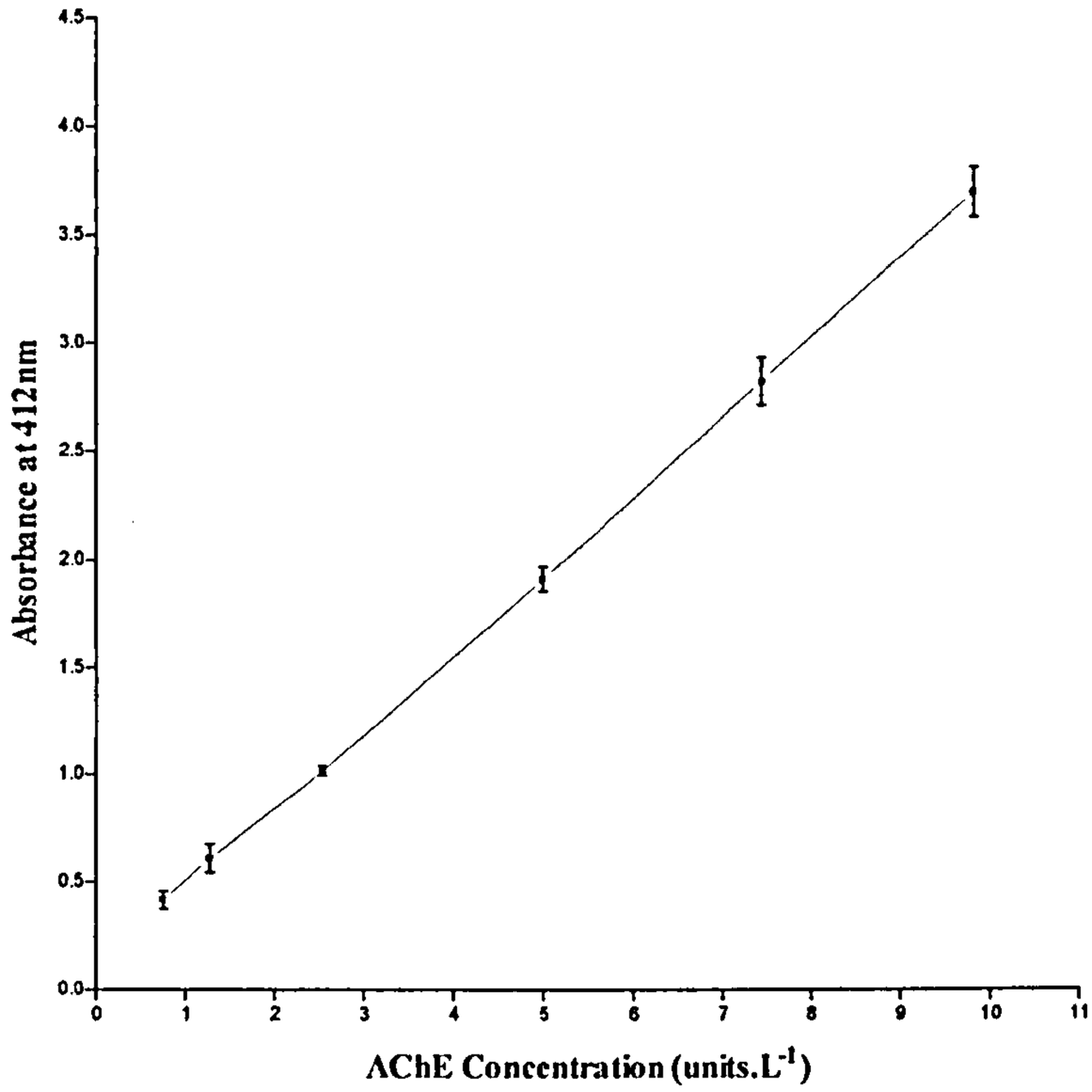


Figure 3.1. Effect of human erythrocyte AChE concentration on the hydrolysis of ATCh (triplicate readings; n=3).

3.2.2 Effect of Plant Extracts and Essential Oils on Erythrocyte AChE Activity

As indicated in Table 3.1, AChE inhibition was apparent with the ethanolic extracts of *Apocynum lancifolium* leaf, *Centella asiatica* leaf, *Convallaria majalis* leaf, *Rosmarinus officinalis* dried leaf, *Salvia miltiorrhiza* root and *Withania somnifera* root; the aqueous extracts of *Apocynum lancifolium* leaf, *Centella asiatica* leaf, *Melissa officinalis* leaf, *Rosmarinus officinalis* dried leaf and *Withania somnifera* root; the petroleum spirit (PS) extract of *Centella asiatica* leaf, and the essential oils from *Melissa officinalis* and *Rosmarinus officinalis*. Extracts from *Apocynum lancifolium*, *Centella asiatica*, *Convallaria majalis*, *Salvia miltiorrhiza* and *Withania somnifera* have not previously been reported to inhibit AChE activity.

Table 3.1. Inhibition of human erythrocyte AChE by plant extracts and essential oils.

Plant Extract or Essential Oil Tested (* Extraction Solvent) ** Adulterated Herb (Substituted with *Gentiana* spp.)	**Concentration (Assay) (µg/ml)**	**Percentage Inhibition of AChE**
Alisma orientalis root (hot EtOH*)	19.0	0
Alisma orientalis root (cold EtOH*)	50.0	0
Alisma orientalis root (hot H_2O*)	9.52	0
***Apocynum lancifolium* dried leaf (cold EtOH*)**	**52.0**	**25.3 ± 1.4**
***Apocynum lancifolium* dried leaf (hot H_2O*)**	**47.6**	**40.1 ± 4.3**
***Centella asiatica* dried leaf (hot EtOH*)**	**50.0**	**39.9 ± 11.5**
***Centella asiatica* dried leaf (hot PS*)**	**30.0**	**42.6 ± 7.1**
Centella asiatica dried leaf (hot H_2O*)	47.6	21.1 ± 3.8
Codonopsis pilulosa root (hot EtOH*)	50.0	0
Codonopsis pilulosa root (cold EtOH*)	310.0	0
Codonopsis pilulosa root (hot H_2O*)	97.0	0
***Convallaria majalis* dried leaf (cold EtOH*)**	**95.2**	**90.9 ± 4.6**
Convallaria majalis dried leaf (hot H_2O*)	95.2	0
***Melissa officinalis* dried leaf (hot H_2O*)**	**47.6**	**81.8 ± 5.7**
***Melissa officinalis* oil (diluted in EtOH)**	**240.0**	**92.7 ± 4.2**
Polygala tenuifolia root (cold EtOH*)	330.0	0
Polygala tenuifolia root (hot H_2O*)	9.52	0
Polygonum multiflorum root ** (hot EtOH*)	19.0	0
Polygonum multiflorum root ** (cold EtOH*)	280.0	0
Polygonum multiflorum root ** (hot H_2O*)	97.0	0
Rosmarinus officinalis fresh leaf (cold EtOH*)	89.0	8.5 ± 4.4
***Rosmarinus officinalis* dried leaf (hot EtOH*)**	**47.6**	**32.3 ± 2.5**
***Rosmarinus officinalis* dried leaf (hot H_2O*)**	**47.6**	**27.2 ± 4.1**

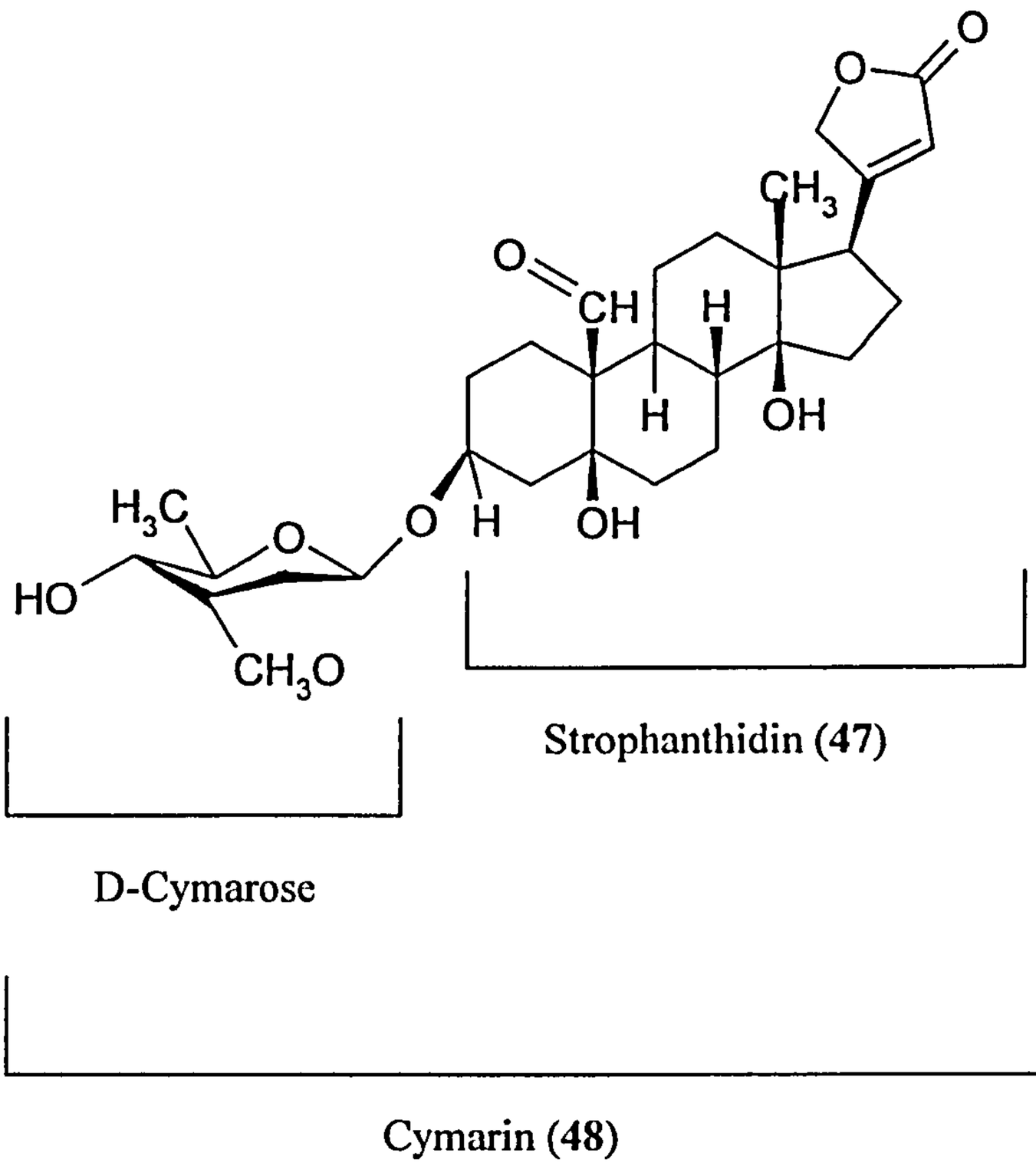
Table 3.1 (continued). Inhibition of human erythrocyte AChE by plant extracts and essential oils.

Plant Extract or Essential Oil Tested (* Extraction Solvent)	**Concentration (Assay) (µg/ml)**	**Percentage Inhibition of AChE**
***Rosmarinus officinalis* oil (diluted in EtOH)**	**240.0**	**85.4 ± 3.8**
***Salvia miltiorrhiza* root (hot EtOH*)**	**19.0**	**26.6 ± 3.9**
***Salvia miltiorrhiza* root (hot EtOH*)**	**38.0**	**42.8 ± 1.7**
***Salvia miltiorrhiza* root (cold EtOH*)**	**190.0**	**61.8 ± 1.25**
***Salvia miltiorrhiza* root (cold EtOH*)**	**370.0**	**83.0 ± 1.25**
***Salvia miltiorrhiza* root (cold EtOH*)**	**Unknown*** **	**51.8 ± 15.1**
Salvia miltiorrhiza root (hot H_2O*)	9.2	12.4 ± 3.5
***Withania somnifera* root (cold EtOH*)**	**70.0**	**38.3 ± 3.5**
***Withania somnifera* root (cold EtOH*)**	**150.0**	**58.2 ± 1.25**
***Withania somnifera* root (cold EtOH*)**	**Unknown*** **	**24.3 ± 6.9**
***Withania somnifera* root (hot H_2O*)**	**47.6**	**55.9 ± 5.4**
Ziziphus jujuba seed (hot PS*)	20.0	0
Ziziphus jujuba seed (hot EtOH*)	50.0	0
Ziziphus jujuba seed (hot H_2O*)	30.0	0
Ziziphus jujuba fruit (cold EtOH*)	50.0	0
Ziziphus jujuba var. *spinosa* seed (hot EtOH*)	97.0	0
Ziziphus jujuba var. *spinosa* seed (hot H_2O*)	97.0	0
Tacrine (positive control)	**$9.5x10^{-5}$M**	**100**

Inhibition is the percentage decrease in activity in the presence of the extract/essential oil compared with the activity in the absence of the extract/essential oil. Results are calculated as the mean ± SD of 3 - 6 different experiments (duplicate readings for each experiment). AChE inhibition > 20% was regarded as active. *** Activity of filtered extract tested prior to evaporating to dryness and reconstituting at a known concentration, to confirm activity before concentrating extract with intention to fractionate.

3.2.2.1 *Apocynum lancifolium*

Apocynum lancifolium leaf and root contain several cardioactive compounds, including the glycosides cymarin (**48**) and K-strophanthin-β (**49**), and the aglycone strophanthidin (**47**) (Huang, 1993; Jianming, 1988). Adverse effects of this herb include nausea, vomiting and diarrhoea (Chang and But, 1987; Huang, 1993). These effects may occur with a variety of drugs, including the cardiac glycoside digoxin. However, these gastro-intestinal disturbances are a frequent adverse effect of the AChE inhibitors donepezil (**8**), galantamine (**9**) and rivastigmine (**6**) so it cannot be excluded that alkaloid AChE inhibitors are present in *A. lancifolium* leaf, and contribute to the adverse effects. Consequently, isolation and identification of active compounds from this herb would be warranted.



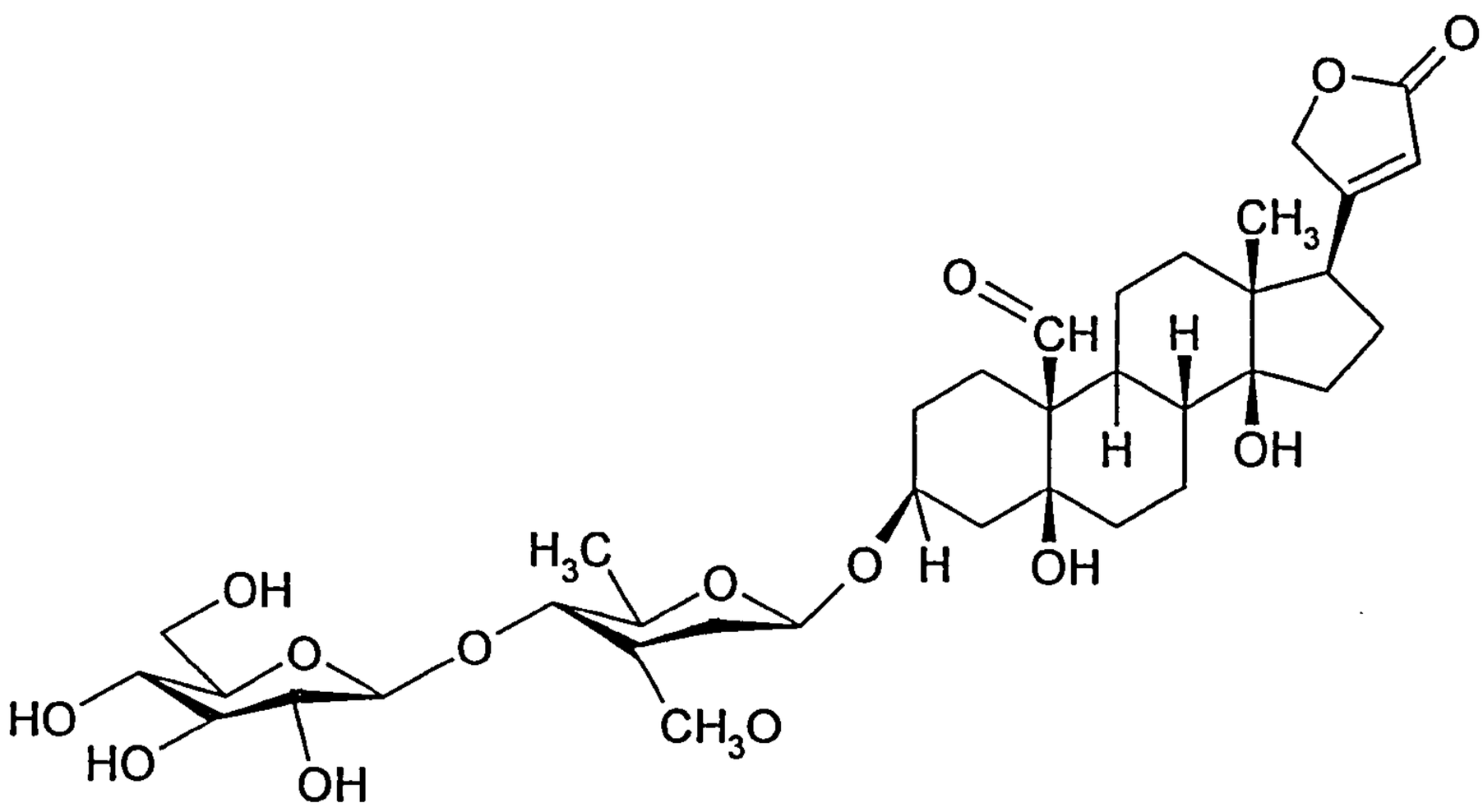
Strophanthidin (**47**)

D-Cymarose

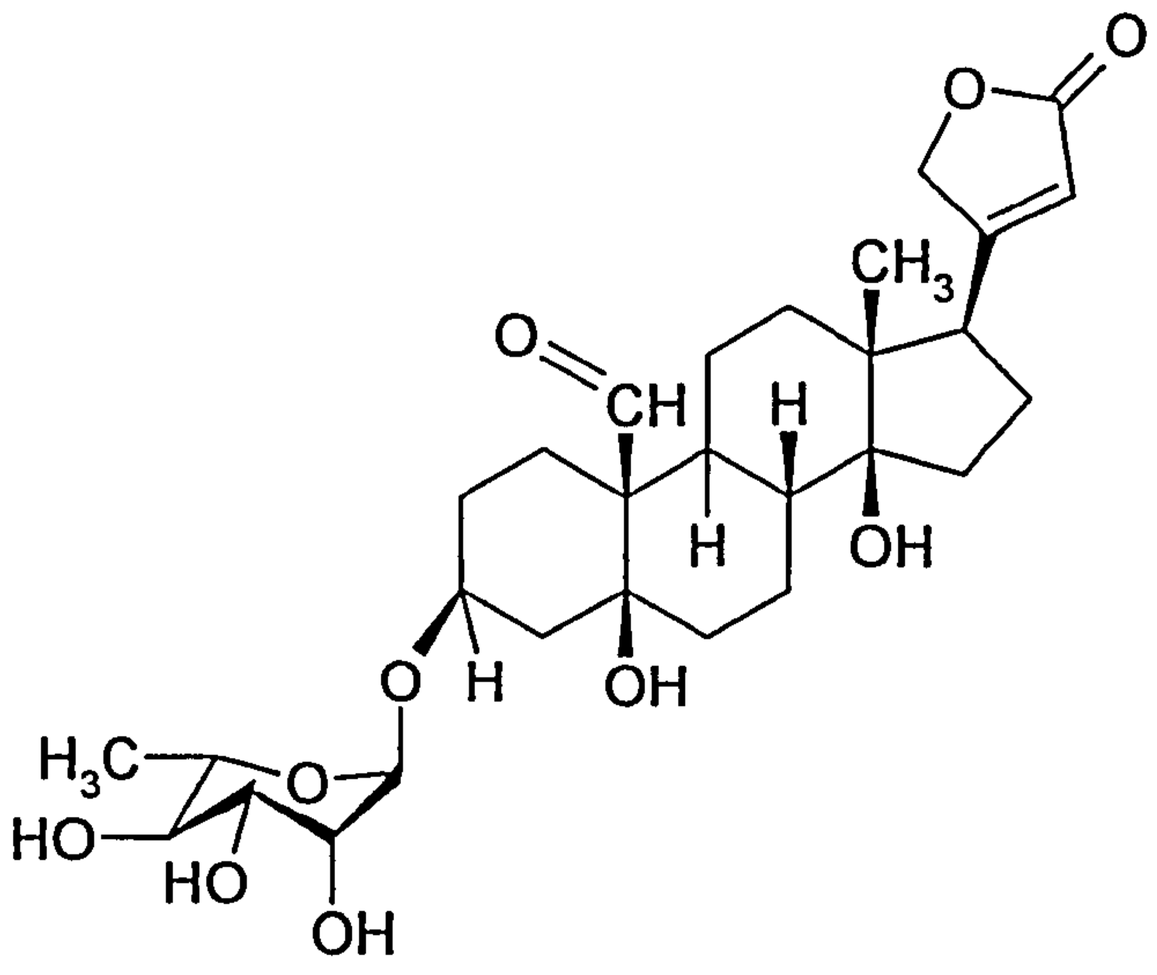
Cymarin (**48**)

Convallaria majalis leaf also contains cardenolide constituents, including the glycosides convallatoxin (**50**), convallamarin, convallarin, convallaric acid, cymarin (**48**), sarhamnoloside and tholloside (Schrutka-Rechtenstamm *et al.*, 1986; Weiss, 1988). Activity against AChE in both these cardenolide containing herbs raises the

possibility that cardenolide compounds may inhibit AChE, if so cardenolides would be a new class of AChE inhibiting compounds, which are quite different in structure from the previously identified AChE inhibitors, alkaloids and monoterpenes.



K-Strophanthin-β (**49**)



Convallatoxin (50)

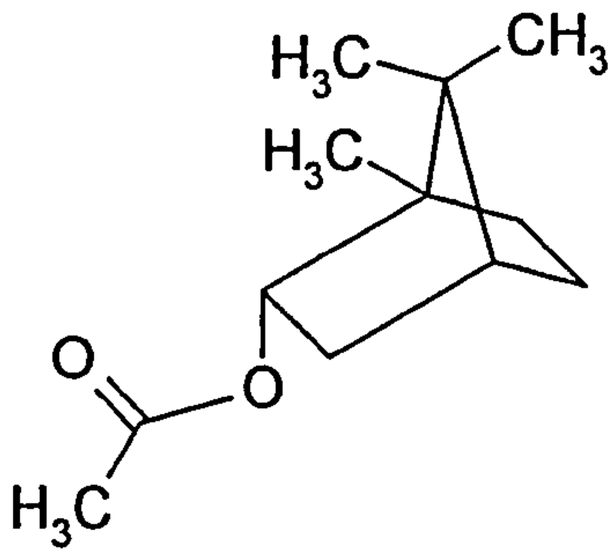
3.2.2.2 *Convallaria majalis*

Convallaria majalis also contains traces of volatile oil (Grieve, 1984), which may indicate the presence of monoterpenes. Assessment of the effect of this volatile oil on AChE activity would be required to determine if it is responsible for the activity observed with the EtOH extract of the herb, or if a synergistic action with other

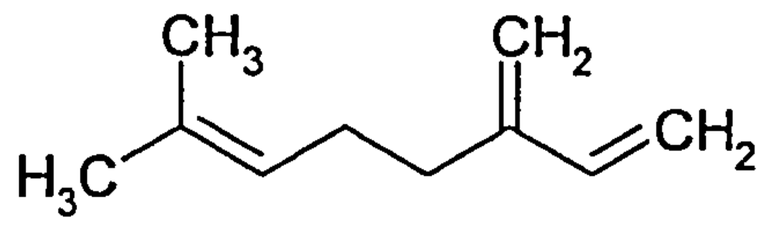
compounds might occur. It is worth noting that the aqueous extract of *C. majalis* (absent of the apolar volatile oil) did not inhibit AChE (Table 3.1). Alkaloids have not been identified in *A. lancifolium* or *C. majalis*, however the possibility of their presence cannot be excluded although it is unlikely. Alkaloids were not detected in the active preparative TLC fractions 3 and 4, F8a (from FCC (a)) and the crude EtOH extract of *C. majalis* herb (refer to Chapter 2, 2.2.2.5). This suggests compounds other than alkaloids in *C. majalis* were antiChE.

3.2.2.3 *Centella asiatica*

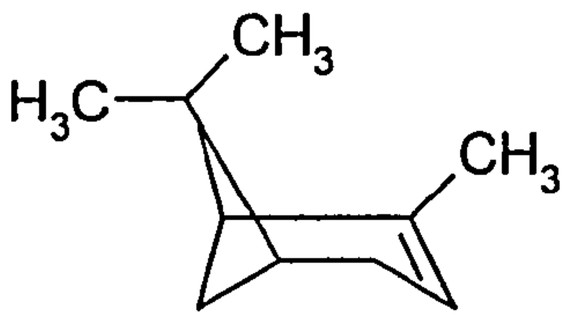
The PS extract of *Centella asiatica* leaf was more active against AChE activity than both the aqueous and EtOH extracts; AChE inhibition was greater in the presence of the PS extract, even though the assay concentration of this extract was lower (Table 3.1). This indicates the constituents responsible for AChE inhibition are relatively non-polar. Monoterpenes, sesquiterpenes and triterpenes are reported to be present in *C. asiatica* leaf (Asakawa *et al.*, 1982; Günther and Wagner, 1996). The monoterpenes present in the essential oil from *C. asiatica* include bornyl acetate (**51**), myrcene (**52**), α-pinene (**53**), β-pinene (**54**) and γ-terpinene (**55**) (Asakawa *et al.*, 1982). These apolar compounds would be expected to be extracted into the more active PS extract, suggesting these monoterpenes may contribute to antiChE activity. Bornyl acetate (**51**) is reported to reversibly inhibit electric eel AChE (Ryan and Byrne, 1988); γ-terpinene (**55**) is an inhibitor of bovine and human erythrocyte AChE (Miyazawa *et al.*, 1997; Perry *et al.*, 2000a). α-Pinene (**53**) is also an inhibitor of erythrocyte AChE, and was a more potent inhibitor than γ-terpinene (**55**); but these monoterpene AChE inhibitors were weak compared to the antiChE alkaloid, physostigmine (**5**) (Perry *et al.*, 2000a). The monoterpenes bornyl acetate (**51**), myrcene (**52**), α-pinene (**53**), β-pinene (**54**) and γ-terpinene (**55**) are reported to comprise less than 2% of the essential oil from *C. asiatica* (Asakawa *et al.*, 1982). With regard to the relative antiChE potency of these monoterpenes, it is improbable that these compounds alone contributed to the antiChE activity of the PS extract.



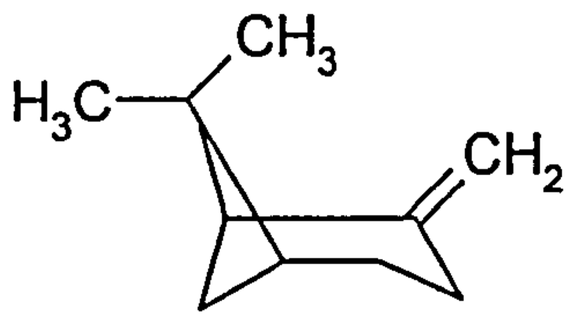
Bornyl acetate (**51**)



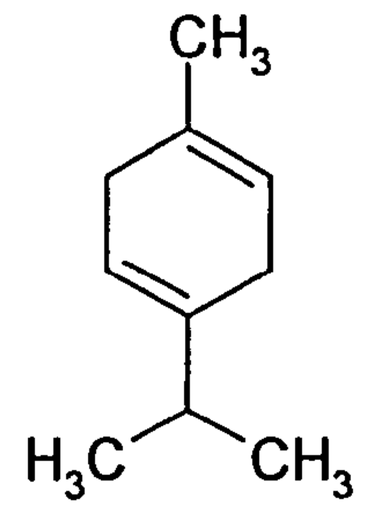
Myrcene (**52**)



α-Pinene (**53**)



β-Pinene (**54**)



γ-Terpinene (**55**)

Other monoterpenes present in the oil may have contributed to activity, as may other unidentified compounds in *C. asiatica* leaf. Alkaloids have been isolated from *C. asiatica*, including the alkaloid hydrocotylin (Duke and Ayensu, 1985). However, the potential antiChE activity of these alkaloids has not been investigated. The alkaloids, the monoterpenes and perhaps other constituents present in *C. asiatica* leaf extracts may have inhibited AChE, perhaps synergistically.

3.2.2.4 *Melissa officinalis* and *Rosmarinus officinalis*

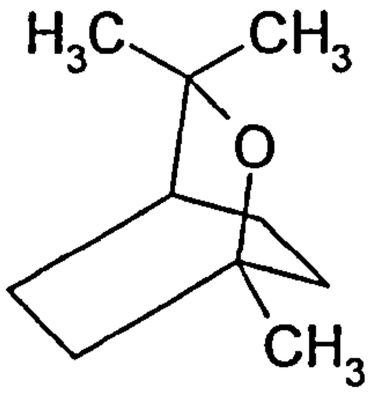
Melissa officinalis herb contains rosmarinic acid (RA) (**108**), glycosidically bound chlorogenic (**107**) and caffeic acids (**106**), triterpenes and flavonoids (e.g. the glycosides of apigenin and luteolin (**114**)) (Bisset, 1994; Bruneton, 1995; Mulkens and Kapetanidis, 1987). It is probable that the activity of the aqueous extract is due to the more polar constituents, perhaps glycoside compounds; the occurrence of significant amounts of the hydrophobic monoterpenes from the essential oil is unlikely in this extract so would not explain antiChE activity.

The hot aqueous and EtOH extracts of *Rosmarinus officinalis* herb caused greater inhibition of AChE, than a higher assay concentration of the cold EtOH extract (Table 1). This suggests that the heating process yielded different compounds from the

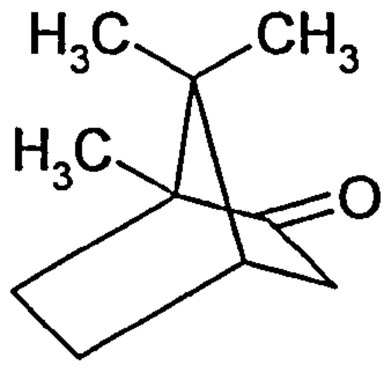
extract, or perhaps thermolabile compounds were converted to antiChE compounds upon heating. As the EtOH extract of the fresh herb was less active than the dried herb, it may be possible that the storage conditions of the dried herb (e.g. humidity, light, temperature) influenced the chemical composition. Alkaloids are not known to be present in either *M. officinalis* or *R. officinalis*. Further research is necessary to isolate and identify active constituents, perhaps alkaloids or novel antiChE compounds.

The essential oils from *M. officinalis* (obtained from G. Baldwin and Co., London and Id Aromatica, Leeds) and *R. officinalis* (obtained from G. Baldwin and Co., London) have previously been reported to inhibit erythrocyte AChE (Perry *et al.*, 1996). Different potencies of an essential oil from the same plant may occur due to several contributing factors; essential oil composition may vary depending on the stage of development and the origin of the leaves, and other factors such as environmental conditions, and time of year harvested. The major constituents of *R. officinalis* essential oil are 1, 8-cineole (**56**) (15-30%), camphor (**57**) (15-25%), α-pinene (**53**) (up to 25%) and other monoterpenes (e.g. borneol (**58**), bornyl acetate (**5**), limonene (**73**)) (Bisset, 1994; Trease and Evans, 1996).

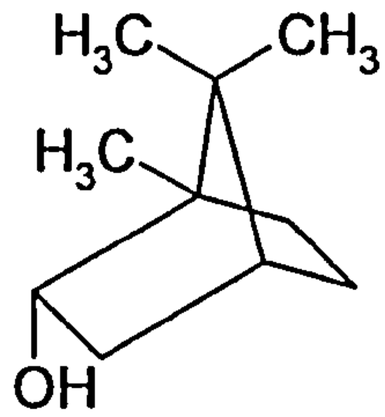
Earlier studies showed that neither 1, 8-cineole (**56**), nor camphor (**57**), nor borneol (**58**) (10^{-4}M) inhibited human erythrocyte AChE (Perry *et al.*, 1996), although 1, 8-cineole (**56**) was shown to inhibit electric eel AChE, the assay concentration being much greater 0.78M (Ryan and Byrne, 1988). More recently, 1, 8-cineole (**56**) and camphor (**57**) have shown activity against human erythrocyte AChE (IC_{50}: 0.63mM and >10mM respectively) (Perry *et al.*, 2000a). The differences observed with the same compound against enzyme inhibition, may be explained by differences in the AChE subtype, the assay method, or the volatility of the monoterpenes at different assay temperatures (higher temperatures may cause loss of the volatile compounds from the assay solution). Limonene (**73**) is also reported to be a weak inhibitor of electric eel AChE (Grundy and Still, 1985).



1, 8-Cineole (**56**)

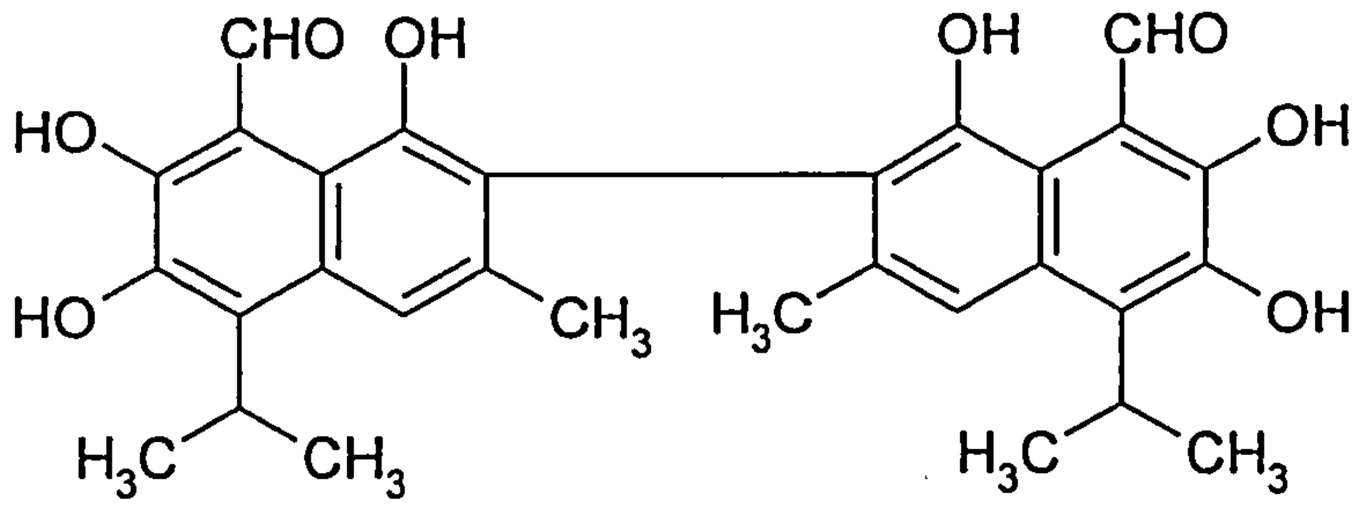


(-)-Camphor (**57**)



(+)-Borneol (**58**)

The *M. officinalis* essential oil used in these investigations includes the constituents citral (geranial (**70**) and neral (**71**)) (11.8% and 8.9% respectively), geraniol (**72**) (0.9%), limonene (**73**) (0.4%), linalool (**74**) (1.2%) and α-pinene (**53**) (0.3%), with the major component being the sesquiterpene *trans*-caryophyllene (**82**) (18.9%) (refer to Chapter 2, 2.2.7.1). Citral is also reported to inhibit electric eel AChE (Ryan and Byrne, 1988), and geraniol (**72**) and linalool (**74**) are weak inhibitors of human erythrocyte AChE (Perry *et al.*, 2000a). It can be concluded that 23.5% of the *M. officinalis* oil used in the present study is composed of known AChE inhibitors. The effect of the other components of these essential oils on AChE activity requires further investigation. A disesquiterpene, gossypol (**59**) is a reversible inhibitor of electric eel AChE (Ryan and Byrne, 1988). This suggests that related sesquiterpene compounds may also be inhibitors of AChE, however the effect of other sesquiterpene compounds, such as *trans*-caryophyllene (**82**), on AChE activity also remains to be established.



Gossypol (**59**)

It may be concluded that the monoterpenes are responsible, at least in part, for the antiChE activity of the essential oils, and may also explain the activity of the EtOH

extracts of *Centella asiatica*, *Convallaria majalis* and *R. officinalis*. However, the structural diversity of the active monoterpenes complicates the prediction of potential structure-activity relationships. One feature associated with enzyme inhibition is a hydrophobic ligand; the hydrophobic active site of AChE is reported to be susceptible to hydrophobic interactions (Hansch and Deutsch, 1966). Monoterpenes consist of a hydrocarbon skeleton, which may explain their antiChE activity. However, no relationship between monoterpene lipophilicity or between molecular volume was identified in the study by Perry *et al.* (2000a). Monoterpenes may be cyclic (e.g. 1, 8-cineole (**56**), and α-pinene (**53**)) or acyclic (e.g. geranial (**70**) and neral (**71**)); a feature that may also influence antiChE activity, but no structure-activity relationship has been established. Further investigations using the same assay method, the same subtype of AChE from the same source, and testing various monoterpenes at the same concentration may determine if a cyclic component, or particular functional group favours AChE inhibition.

The nature of enzyme inhibition may also affect the potency of a ligand. For example, an inhibitor may bind competitively (competing with the substrate for the active site) or non-competitively (affecting the AChE-ATCh complex, preventing product formation); a competitive inhibitor may interact with one or more subsites (the anionic, hydrophobic and esteratic sites) in the active site, or may influence a completely different site on the enzyme and so alter the molecular configuration, rendering the enzyme inactive.

3.2.2.5 *Salvia miltiorrhiza*

Salvia miltiorrhiza root (EtOH extract) was significantly active against AChE activity at the concentrations tested, however the aqueous extract was not (Table 3.1). This gives some indication of the type of compounds that are antiChE. The less polar diterpene compounds (e.g. tanshinone I (**115**), dihydrotanshinone, tanshinone IIa (**116**) and cryptotanshinone (**117**)), which have previously been isolated from the lipophilic extracts of *S. miltiorrhiza* root (Paulus and Bauer, 2000), are likely to be present in the EtOH extract, rather than the less active aqueous extract. It is therefore possible that the lipophilic components, perhaps the diterpenes, of *S. miltiorrhiza* root are more potent inhibitors of AChE than the more polar components. To confirm this

observation, the EtOH and aqueous extracts were also assessed over the concentration range 5.8µg/ml - 70.9µg/ml (refer to 3.2.3).

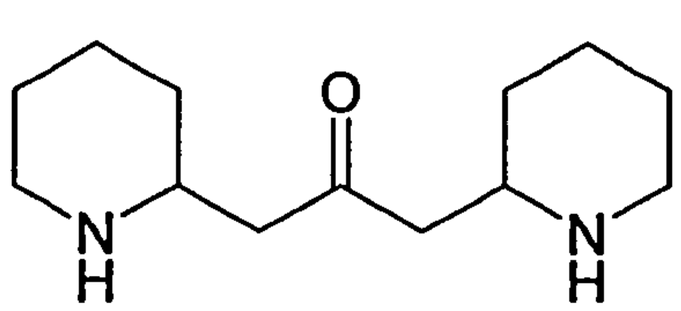
3.2.2.6 *Withania somnifera*

A lower concentration (47.6 µg/ml) of the aqueous extract of *Withania somnifera* root was more active against AChE activity (55.9% inhibition) than a higher concentration (70.0µg/ml) of the EtOH extract (38.3% inhibition) (Table 3.1). This indicates that AChE inhibitors are present in both extracts, but those in the aqueous extract may be more potent, or act synergistically to enhance inhibitory activity.

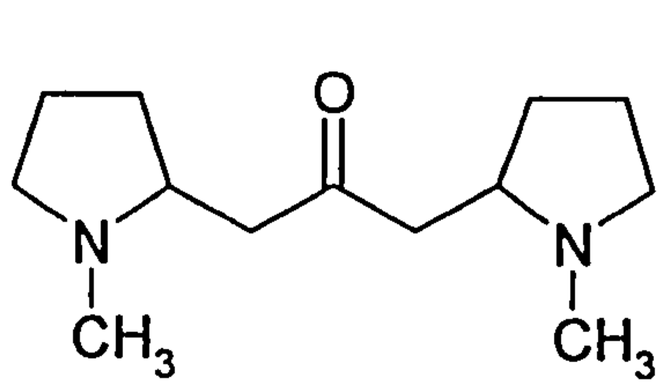
Numerous alkaloids have been identified in *W. somnifera* root; these include anaferine (**60**), anahygrine, ashwagandhine, aswhagandhinine, cuscohygrine (**61**), isopelletierine, pseudotropine, pseudowithanine, somniferiene, somniferinine, tropine (**62**), visamine, withanine, withaninine and withasomine (Harborne and Baxter, 1993; Mills and Bone, 2000; Schwarting *et al.*, 1963; Upton, 2000). Nicotine (**2**) is reported to be present in *W. somnifera* root (Kapoor, 1990) but some investigations have not detected its presence (Das *et al.*, 1963; Schwarting *et al.*, 1963). This may be explained by variation in the source of the herb, as chemical composition may be influenced by several factors, including environmental conditions for cultivation and storage conditions, or by different extraction procedures used to isolate chemical constituents.

It remains to be determined whether any of the alkaloids (perhaps those yet to be identified) present in the root influence AChE activity. An alkaloid containing extract from *W. somnifera* has shown tranquillising effects on the CNS *in vivo* (Malhotra *et al.*, 1965), however effects of *Withania* alkaloids on cognitive function remain to be investigated. The various alkaloids obtained from plant sources (e.g. galantamine (**9**), huperzine A (**10**), physostigmine (**5**)) which are known AChE inhibitors, leads to the suggestion that the alkaloidal content of *W. somnifera* root is responsible for the antiChE effects observed, however the possibility of non-alkaloidal compounds from the root inhibiting AChE cannot be excluded. *Withania* alkaloids differ in their chemical structures, and include piperidine alkaloids (e.g. anaferine (**60**), anahygrine, cuscohygrine (**61**)) and tropane alkaloids (e.g. tropine (**62**)). Some tropane alkaloids (e.g. hyoscine (**63**) and hyoscyamine (**64**)) are reported to be anticholinergic (Harborne and Baxter, 1993), and consequently induce amnesia. Potential

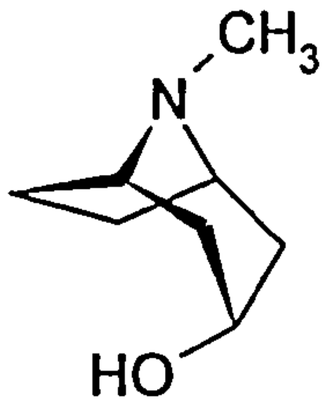
anticholinergic effects of the alkaloids from *W. somnifera* remain to be investigated, however the reputed benefits on memory in traditional medicine, suggest that any possible amnesic effects are counteracted by cognitive enhancement.



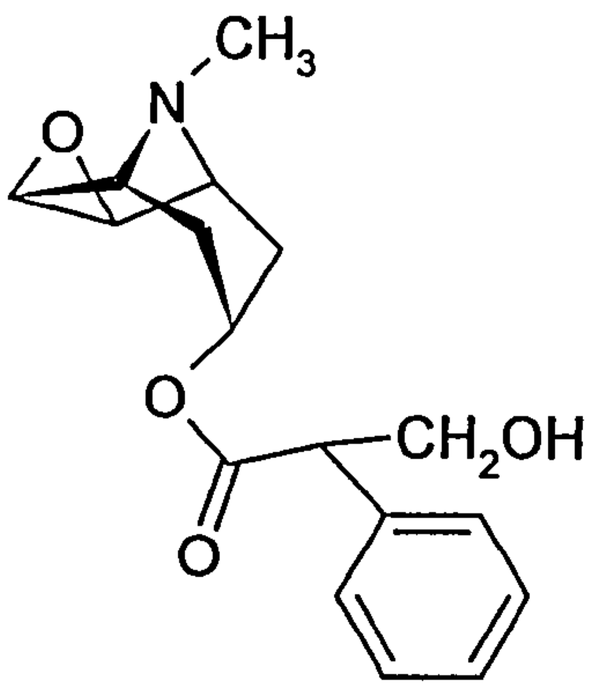
Anaferine (**60**)



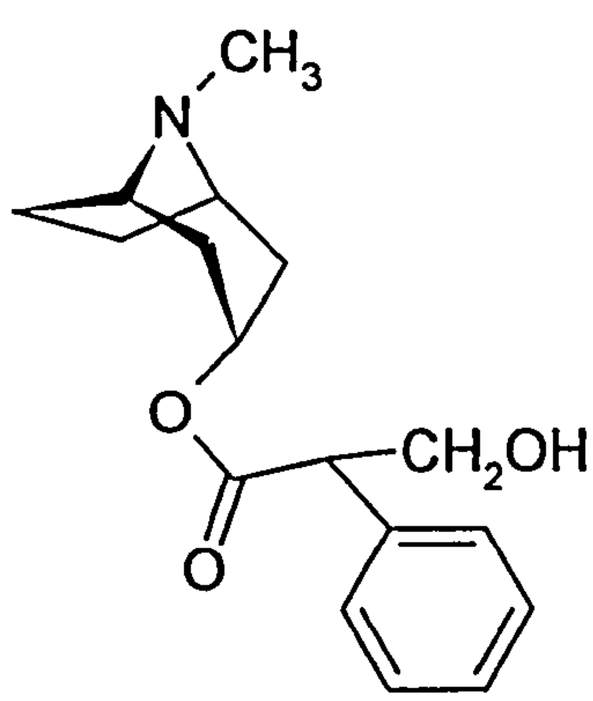
Cuscohygrine (**61**)



Tropine (**62**)



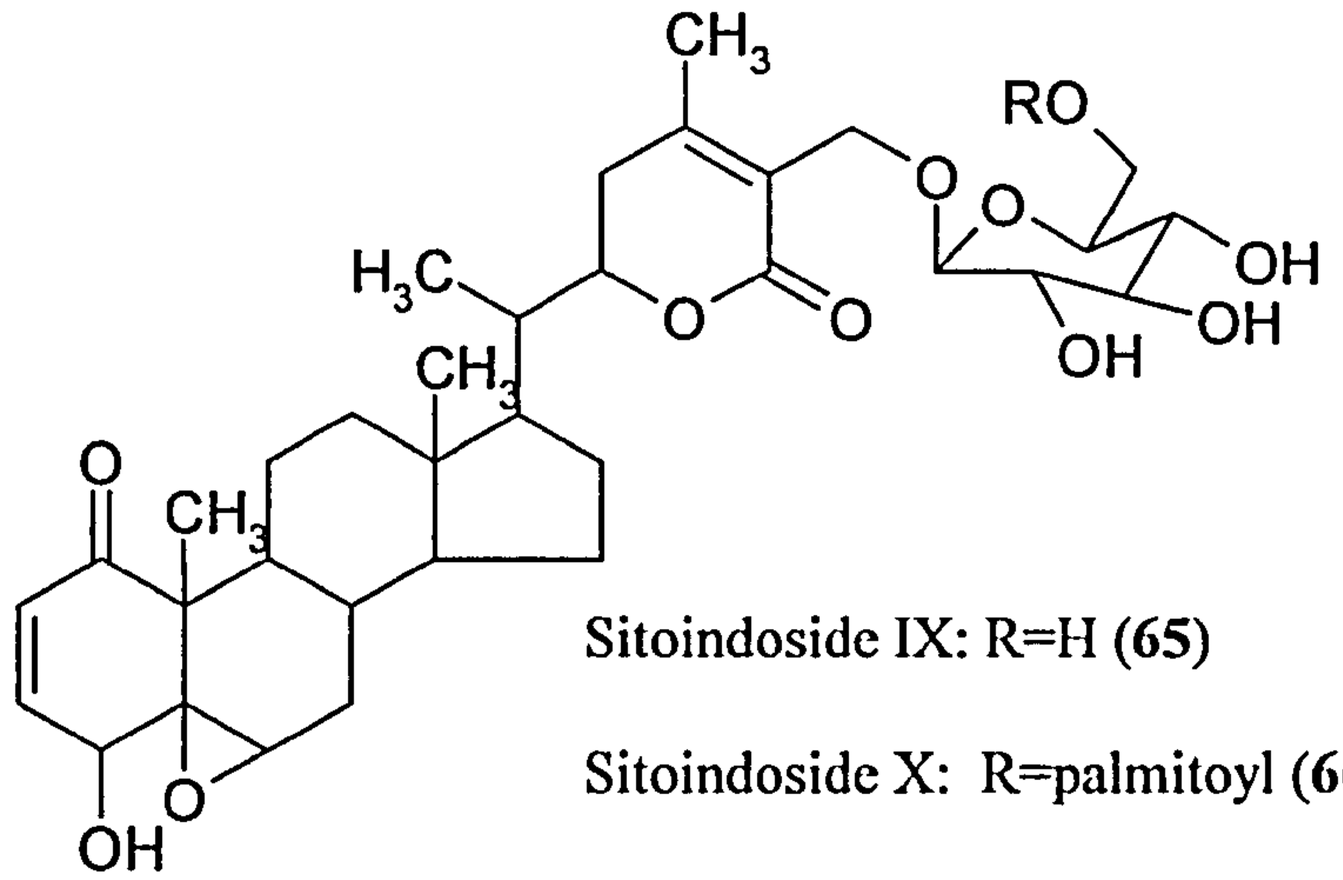
Hyoscine (**63**)



Hyoscyamine (**64**)

It cannot be predicted if the particular alkaloids from *W. somnifera* are antiChE, as structure activity relationships to determine affinity for the AChE enzyme have not been established; those alkaloids which are known AChE inhibitors vary in their chemical structure, for example galantamine (**9**) is a phenylalanine-derived alkaloid, berberine, palmatine and sanguinarine are isoquinoline alkaloids, physostigmine (**5**) is an indole alkaloid and solanine is a steroidal alkaloid.

It is reported that an extraction with 45% alcohol yields the highest percentage of alkaloids from *W. somnifera* root (Kapoor, 1990). Comparison of the antiChE activity of such an extract, with the antiChE activity of the aqueous and EtOH extracts may yield further information regarding the potential antiChE activity of the *W. somnifera* alkaloids; isolation and screening of each alkaloid for antiChE activity may also explain the activity of the crude extracts.



Sitoindoside IX: R=H (**65**)

Sitoindoside X: R=palmitoyl (**66**)

Several steroidal derivatives have also been isolated from *W. somnifera* root, including the withanolide aglycones (e.g. withanolides A - Y, withaferin A (**109**)) and the glycosylated sitoindosides (Ghosal *et al.*, 1989; Mishra *et al.*, 2000; Rhaman *et al.*, 1991; Rhaman *et al.*, 1993). It is these compounds that are believed to be responsible for the optimum improvement in cognitive function in several *in vivo* studies. Sitoindoside IX (**65**) and X (**66**) augmented learning acquisition and memory retention in young rats; however these observations were more significant in old rats (Ghosal *et al.*, 1989), suggesting therapeutic relevance in the aged brains of many AD patients. Withaferin A (**109**) was not shown to initiate this cognitive enhancement (Ghosal *et al.*, 1989), suggesting inactivity against cognitive dysfunction, or that synergism (perhaps with the sitoindosides) is a requirement for activity. The mechanism of action to explain these observations was undetermined, however the greater effects in the aged rat brain indicate that activity is greatest under suboptimum cholinergic function. It is possible that in the aged or diseased (AD) brain, degeneration of cholinergic neurons may be compensated for by an increased sensitivity of cholinergic receptors, thus promoting cholinergic function.

Further studies have shown that *W. somnifera* root extracts, consisting of equimolar amounts of sitoindosides VII - X and withaferin A (**109**), significantly reversed an ibotenic acid (IA) induced cognitive deficit and the reduction in cholinergic markers (ACh (**1**), ChAT, muscarinic receptors) *in vivo* (Bhattacharya and Kumar, 1995). These findings may explain the earlier observations on cognitive function. More recently the effects of an extract containing equimolar amounts of sitoindosides VII - X and withaferin A (**109**), on cholinergic markers, have been investigated (Schliebs *et*

al., 1997). It was found that AChE activity was enhanced in the lateral septum and globus pallidus, but activity was decreased in the diagonal band in rat brain, following systemic administration; in addition, M_1 muscarinic receptor binding was enhanced in the lateral and medial septum, and in the frontal cortices, and M_2 receptor binding in various cortical regions including the frontal and parietal cortices (Schliebs *et al.*, 1997). These results suggest compounds in the extract preferentially affect cholinergic function in the basal and cortical forebrain, which are areas in the brain important for cognitive function. It is proposed that the changes in AChE activity observed in the study by Schliebs *et al.* (1997) may be due to loss of cholinergic neurons, suppression of AChE expression in neuronal cell bodies, or direct inhibition of the AChE enzyme. The *in vitro* effects against AChE activity, observed in the present study, confirm that *W. somnifera* root is an inhibitor of AChE, but this does not exclude the occurrence of additional mechanisms of action *in vivo*.

W. somnifera is also reported to contain choline (Kapoor, 1990), a precursor of ACh (1). The presence of choline in systemically administered extracts may have contributed to the cognition enhancing effects observed in the *in vivo* studies, perhaps by acting synergistically with other compounds. The *W. somnifera* root extract both enhanced and decreased AChE activity in different brain regions (Schliebs *et al.*, 1997), this may be due to compounds being either enzyme inducers or inhibitors, depending on the subtypes of AChE present in a particular brain region. As the extract, which influenced AChE activity *in vivo*, consisted of the sitoindosides VII - X and withaferin A (**109**), it is apparent that these compounds and perhaps related compounds may be responsible for the antiChE effects observed *in vitro* in the present study.

3.2.3 Effect of *Salvia miltiorrhiza* Root Extracts on Erythrocyte AChE Activity

The EtOH extract of *Salvia miltiorrhiza* root was confirmed to inhibit erythrocyte AChE more effectively than the aqueous extract, when tested over the concentration range 5.8µg/ml - 70.9µg/ml (Figure 3.2). The IC_{50} value for the EtOH extract was 37.2µg/ml; the aqueous extract inhibited AChE by 45% at the maximum assay concentration (IC_{50} <50%).

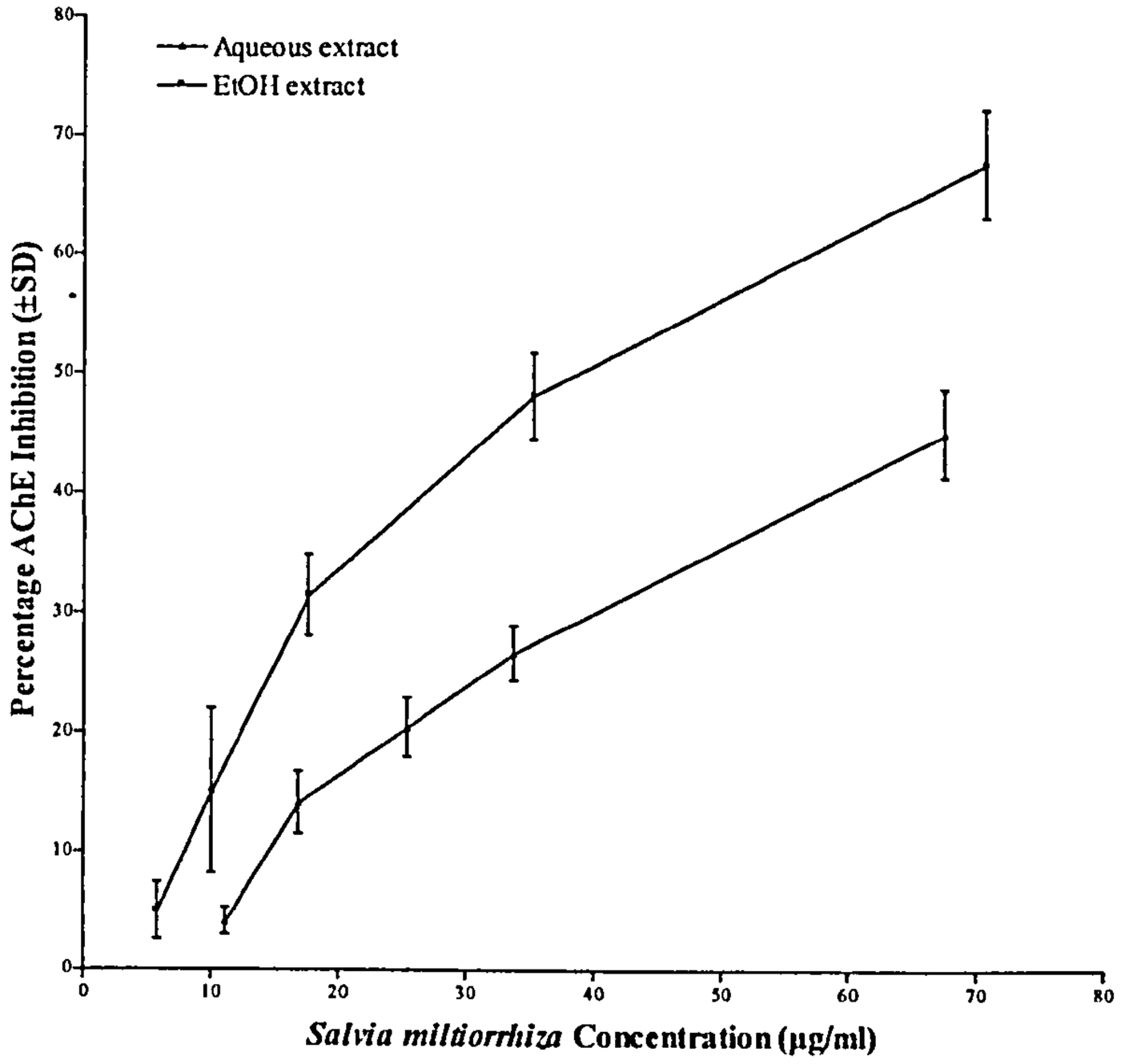


Figure 3.2. Inhibition of erythrocyte AChE activity by aqueous and ethanolic extracts of *Salvia miltiorrhiza* root (duplicate readings; n=3; $p<0.05$).

Other *Salvia* spp. have been reported to inhibit AChE, including the essential oil from the leaves of *S. officinalis*, and the and the leaves and essential oil from *S. lavandulifolia* (Perry *et al.*, 1996; Perry *et al.*, 2000a). In these studies, the antiChE activity of the *S. lavandulaefolia* leaf extract was attributed to the essential oil content. The root of *S. miltiorrhiza* is quite different in chemical composition to the leaf essential oil of other *Salvia* spp.; *S. miltiorrhiza* root contains diterpene pigments with a phenanthrenequinone structure (e.g. tanshinones and isotanshinones) as the major components, whereas monoterpenes are the major constituents of *S. officinalis* and *S. lavandulaefolia* essential oils.

The antiChE activity of both the aqueous and EtOH extracts of *S. miltiorrhiza* root indicate that different compounds are responsible for the inhibitory activity, and that they are novel antiChE compounds from the genus *Salvia*. The EtOH extract would be expected to contain the less polar constituents, such as the diterpene tanshinone compounds, and the aqueous extract would be expected to contain water soluble compounds as the major components, such as the phenylpropanoids (e.g. danshensu (**45**)) and salvianolic acids A (**123**), B (**124**) and C (**125**) (reported to be isolated from an aqueous extract of *S. miltiorrhiza* root (Chen and Liu, 1980; Tang and Eissenbrand,

1992)). It is feasible that these compounds or other unidentified compounds are novel inhibitors of AChE.

3.2.4 Effect of *Convallaria majalis* Leaf Extracts, Fractions and Known Constituents on Erythrocyte AChE Activity

3.2.4.1 Effect of *C. majalis* Leaf Extracts (Hexane, Dichloromethane, Ethanol and Water Extracts) on Erythrocyte AChE Activity

Of the *C. majalis* extracts tested (95.24µg/ml), only the DCM and EtOH extracts were significantly active against AChE activity, giving 82.0% and 90.9% inhibition respectively (Figure 3.3).

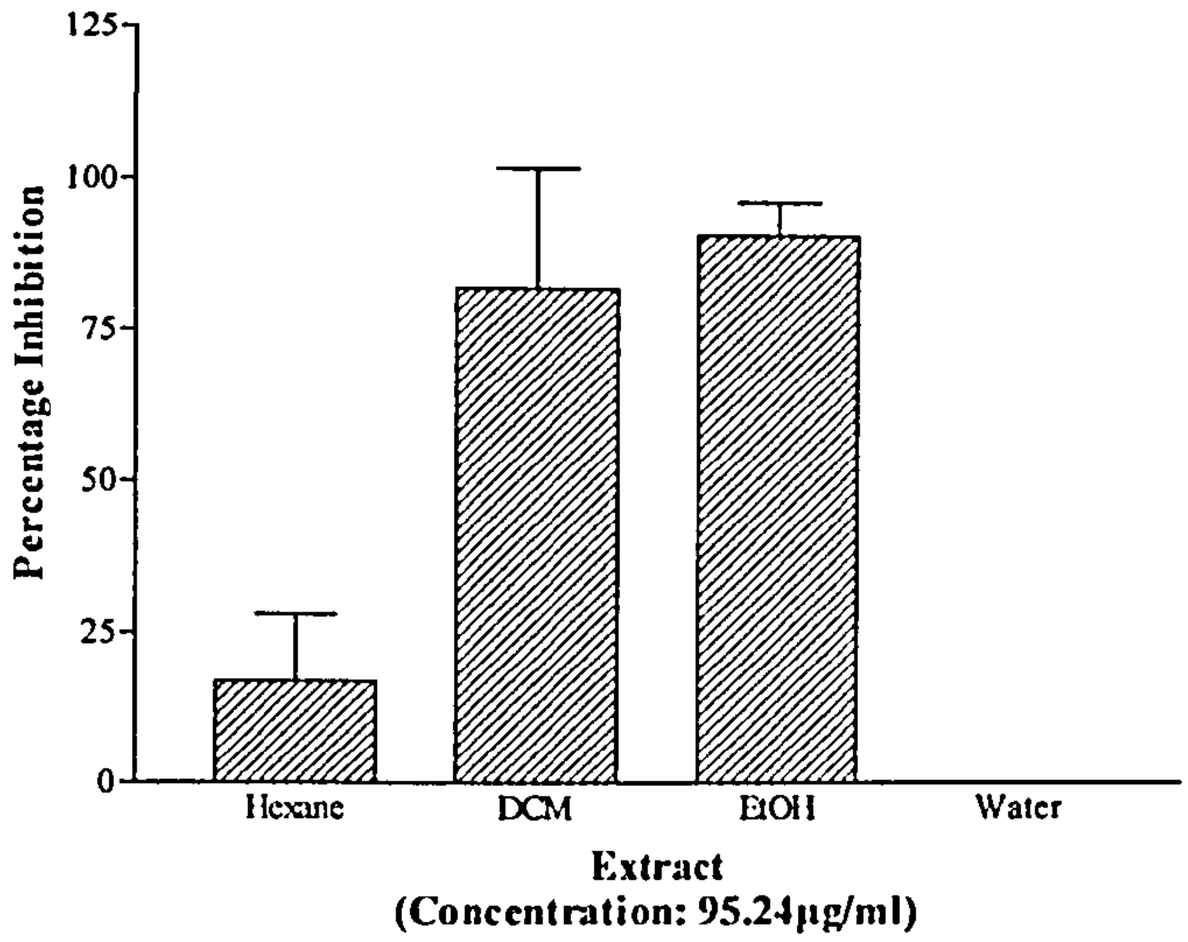


Figure 3.3. Inhibition of erythrocyte AChE activity by extracts from *C. majalis* leaf (duplicate readings; n=3 ± SD).

These results indicate that compounds in *C. majalis* leaf that are highly polar, and also those that are less polar are not significantly active against AChE activity. However, it cannot be excluded that a minor component of these extracts is a potent AChE inhibitor, and may have been present at a concentration too low to influence AChE. TLC analysis showed that the EtOH and DCM extracts have a similar chemical profile (refer to Chapter 2, 2.2.4), which may explain their high activity, compared to

the less active aqueous and hexane extracts. *C. majalis* leaf is reported to contain cardiac glycosides, flavonoid glycosides and the less polar aglycones of these compounds (Trease and Evans, 1996). The presence of some glycosides of these compounds would be expected in the more polar extracts (aqueous and EtOH extracts), and the corresponding aglycones in the less polar extracts (C_6H_{14}, DCM and perhaps EtOH). It may be these compounds or perhaps other unidentified compounds are active against AChE. LC-MS analysis of the crude EtOH extract showed some compounds to be present which appeared to be glycosides (refer to Chapter 2, 2.2.3). These may have been cardiac or flavonoid glycosides, but were not found to be either convallatoxin (**50**) or cymarin (**48**), suggesting these compounds were absent in the EtOH extract. These glycosides may have been antiChE, but to establish this, their isolation is necessary.

3.2.4.2 Effect of Flash Column Chromatography Fractions from *C. majalis* Leaf Ethanol Extract (a) and Pure Compounds on Erythrocyte AChE Activity

Of the sixteen fractions (refer to Chapter 2, 2.1.6.1 for fractionation method) analysed for activity against AChE, only four fractions were significantly active, i.e. F4a, F8a, F9a and F16a inhibited AChE activity by 33.5%, 34.2%, 24.7% and 34.5% respectively (Figure 3.4). All other fractions inhibited AChE activity by <20%.

Flavonoid compounds were not detected in the fractions, but one zone likely to be due to a flavonoid was detected following TLC analysis of the crude EtOH extract (refer to Chapter 2, 2.2.2.1). It may be concluded that the apparent absence of flavonoids in the active fractions suggests these compounds are not AChE inhibitors. Flavonoids are not known to be antiChE compounds. The pure compound hyperoside, which is a flavonol *O*-glycoside, (flavonoid glycosides are reported to be present in *C. majalis* (Trease and Evans, 1996)) did not inhibit AChE (Figure 3.4), providing further evidence for the inactivity of flavonoids against AChE. To confirm these results other classes of flavonoid compound could be tested for inhibition of AChE.

Unidentified cardenolide compounds were detected in F3a and F8a (refer to Chapter 2, 2.2.1.1). Cardenolides are not typically associated with inhibition of AChE. F3a only inhibited AChE by 16.4%, being less active than F8a. It may possible that cardenolide compounds present in these fractions are AChE inhibitors, either alone or in synergy with other compounds. The cardenolide in F3a may be less active than the

cardenolide detected in F8a, or may be more potent than the F8a cardenolide, but present at a lower concentration.

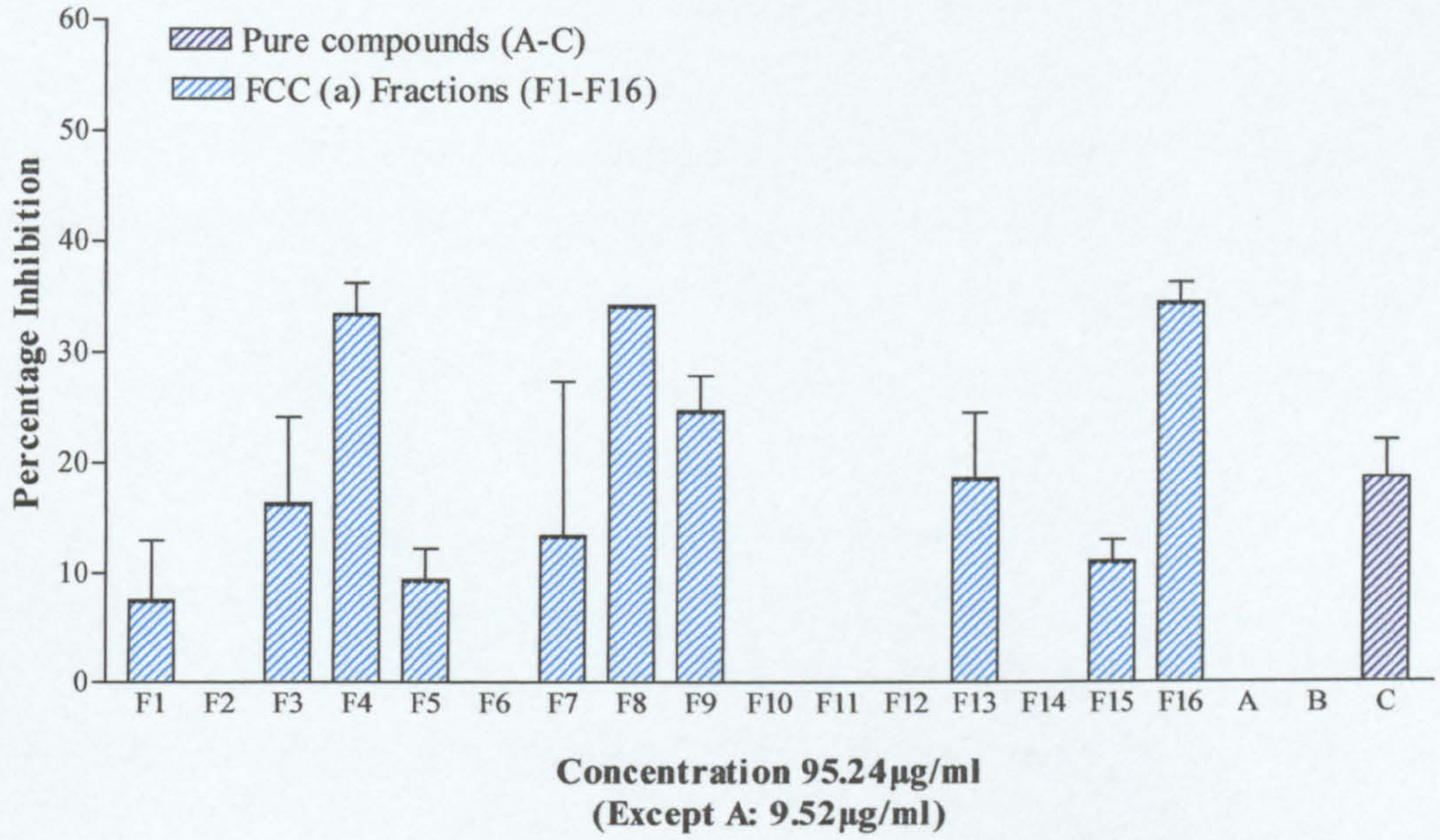


Figure 3.4. Inhibition of erythrocyte AChE by flash column chromatography fractions (FCC (a)) of *C. majalis* leaf (ethanol extract) and pure compounds (duplicate readings; n=3 ± SD). A = hyperoside, B = convallatoxin, C = cymarin.

To provide further information regarding the potential antiChE activity of cardenolides, two pure cardenolide compounds were assessed, convallatoxin (**50**) (reported to be the major cardenolide compound present in *C. majalis* (Harborne, 1993)) and cymarin (**48**). Of the pure compounds tested, only cymarin (**48**) inhibited AChE activity, but inhibition was not significant (18.9%) (Figure 3.4). This suggests that some cardenolide compounds may be weak inhibitors of AChE, and may explain the antiChE activity of the herb and subsequent fractions, perhaps by synergism with other compounds. The antiChE activity of cymarin (**48**) suggests some cardenolides are novel inhibitors of AChE; however further investigations are required to determine the antiChE potential of other cardenolides, and any structure-activity relationships. The cardenolides do not contain the nitrogen in a ring system, which is a feature of the antiChE alkaloids, nor are they small hydrocarbons as are the antiChE monoterpenes.

It is worth noting that a cholinergic link has been identified in the genesis of cardiac arrhythmias (David *et al.*, 1982), which may aid the understanding of the potential antiChE activity of cardenolides. It has been shown that arrhythmogenic agents (e.g. K-strophantin) induce ACh (**1**) release, with the exception of ACh (**1**) in the presence of physostigmine (**5**) (David *et al.*, 1982). Previous work by Schliebs *et al.* (1997), showed that *Withania somnifera* extracts may enhance or reduce AChE activity in different brain regions. This occurrence may be due to compounds acting as enzyme inducers or inhibitors, depending on the subtype of AChE. Similarly, a cardenolide may act as an enzyme inducer to initiate arrhythmias, but may act as enzyme inhibitor if the enzyme subtype were different, such as the AChE subtypes in the CNS. This theory would support the finding that some cardenolides may inhibit AChE since cardiogenic agents may influence ACh (**1**) release (David *et al.*, 1982), and if this effect were to occur in the CNS, then cholinergic function may be enhanced. This effect may also explain the reputed beneficial effects of *C. majalis* leaf on memory.

3.2.4.3 Effect of Flash Column Chromatography Fractions from *C. majalis* Leaf (Dichloromethane Layer of an Ethanol Extract) (b) on Erythrocyte AChE Activity

Nine of the seventeen fractions (refer to Chapter 2, 2.1.6.2 for fractionation method) tested were significantly active against AChE activity; F1b, F3b, F5b, F6b, F7b, F10b, F11b, F14b and F15b inhibited AChE activity by 23.2%, 40.6%, 33.6%, 23.0%, 24.2%, 36.4%, 70.4%, 21.1% and 22.6% respectively. All other fractions inhibited AChE activity by <20% (Figure 3.5).

F1b was a non-polar (PS) fraction, indicting that active compounds were non-polar, and different to those active compounds present in the more polar F15b (DCM : MeOH (1:1) fraction). It is therefore apparent that antiChE activity of *C. majalis* leaf is due to more than one type of compound. F11b showed greatest inhibition of AChE (70.4%) of all the FCC (b) fractions tested. This fraction was obtained from the solvent gradient DCM : MeOH (6:1 to 3:1). The presence of some of these compounds in F10b, may explain the antiChE activity of this fraction, although other perhaps less potent compounds may have contributed to activity. Compounds present in F10b and F11b (from FCC (b)), were not apparent in F15a, or the more active F16a (from FCC (a)) (refer to Chapter 2, 2.2.2.3) suggesting different compounds in *C. majalis* leaf are antiChE. Several compounds in the active F8a (from FCC (a)) were

also present in F6b and F7b (from FCC (b)) (refer to Chapter 2, 2.2.2.3), perhaps being responsible for antiChE activity in all three active fractions.

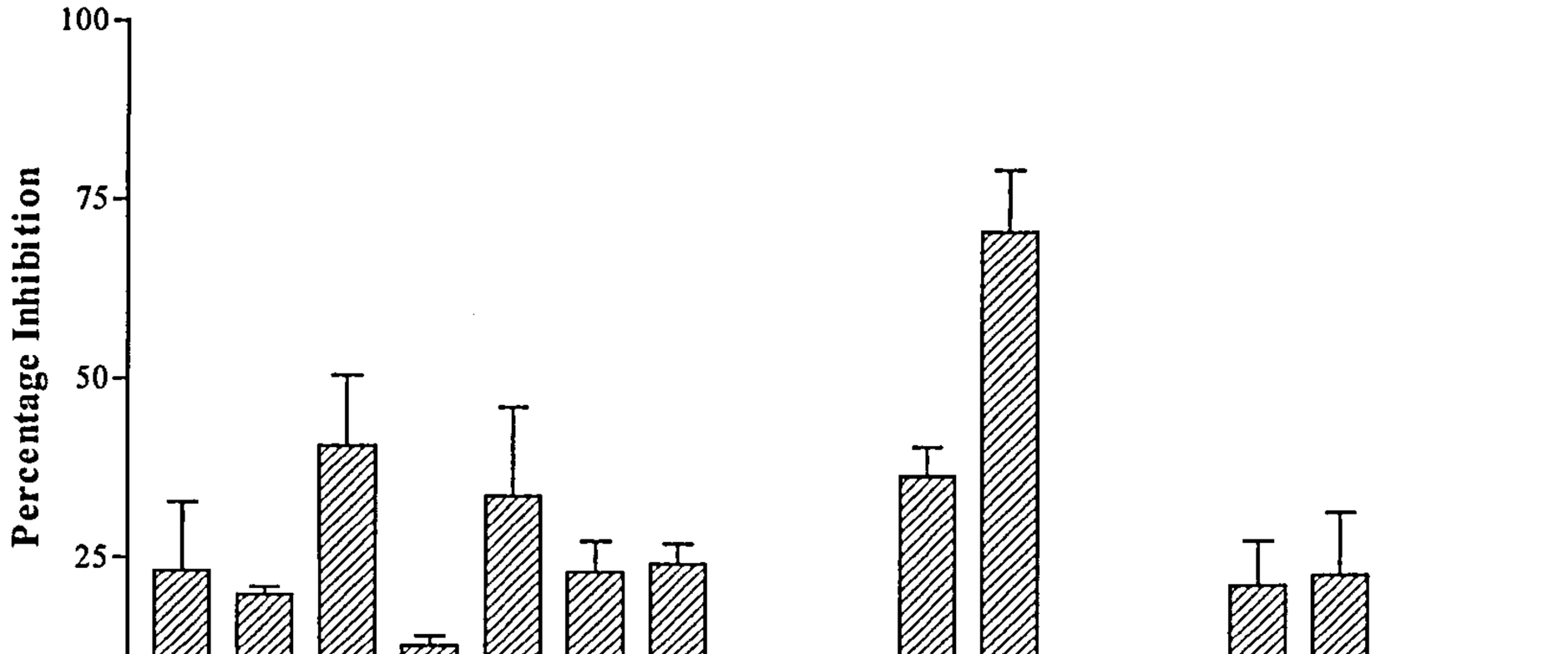


Figure 3.5. Inhibition of erythrocyte AChE by flash column chromatography fractions (FCC (b)) of *C. majalis* leaf (dichloromethane layer of an ethanol extract) (duplicate readings; n=3 ± SD).

3.2.4.4 Effect of Droplet Counter-Current Chromatography Fractions from *C. majalis* Leaf Ethanol Extract on Erythrocyte AChE Activity

Six of the seventeen fractions (refer to Chapter 2, 2.1.6.3 for fractionation method) tested were significantly active against AChE activity. These fractions were F2, F4, F8, F9, F13 and F17, which inhibited AChE activity by 23.0%, 89.6%, 59.1%, 64.6%, 31.2% and 31.6% respectively (Figure 3.6). All other fractions inhibited AChE activity by <20%.

Almost 90% inhibition of AChE occurred in the presence of F4 (a fraction obtained by pooling fractions 62 - 71 from the original 459 DCCC fractions), which was composed of both polar and more lipophilic compounds (refer to Chapter 2, 2.2.2.4). The three TLC zones identified in the active F8a (from FCC (a)), and the preparative TLC F3 and F4 (refer to Chapter 2, 2.2.2.5) were not identified in DCCC F4 (refer to

Chapter 2, 2.2.2.4), which suggests that there are compounds present in *C. majalis* leaf, other than those in these three zones, which are potent inhibitors of AChE.

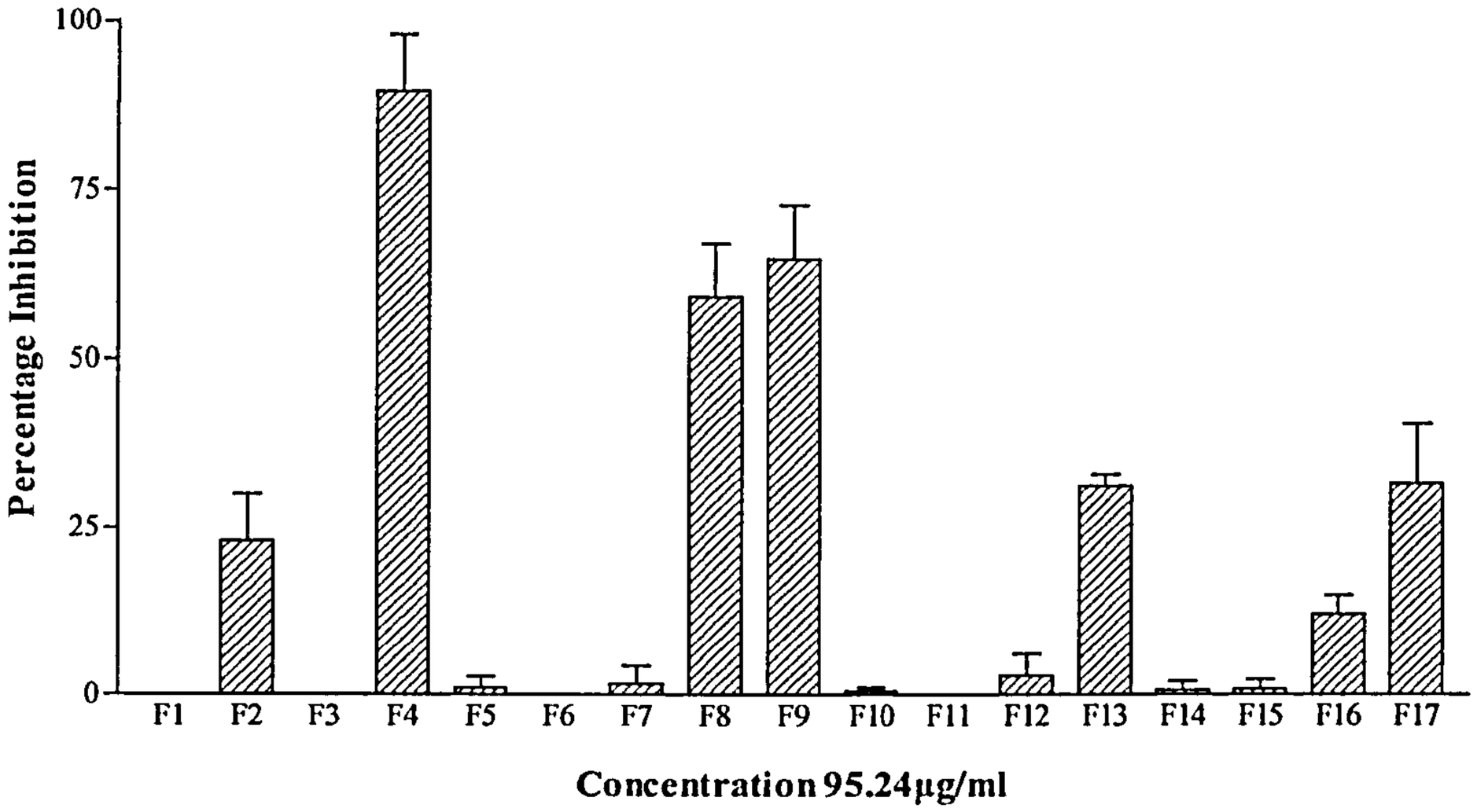


Figure 3.6. Inhibition of erythrocyte AChE by droplet counter-current chromatography fractions of *C. majalis* leaf (ethanol extract) (duplicate readings; n=3 ± SD).

3.2.4.5 Effect of Preparative Thin Layer Chromatography Fractions from *C. majalis* Leaf Ethanol Extract on Erythrocyte AChE Activity

Four fractions from the preparative TLC separation of *C. majalis* EtOH extract (refer to Chapter 2, 2.1.7.8 for fractionation method) were significantly active against AChE activity; F2, F3, F4 and F7 inhibited AChE activity by 35.4%, 46.4%, 48.5% and 46.9% respectively (Figure 3.7). All other fractions inhibited AChE activity by <20%.

Following TLC analysis, three zones were found to be present in F3 and F4, and in F8a (from FCC (a)) and also in the crude EtOH extract of *C. majalis* leaf (refer to Chapter 2, 2.2.2.5). These compounds may therefore explain the antiChE activity of all three fractions, and the crude extract. Alkaloids were not detected in these three zones and F8a (from FCC (a)) was negative for the presence of flavonoids (refer to Chapter 2, 2.2.2.1, 2.2.2.5). HPLC analysis of F3 also showed the absence of

flavonoid compounds (refer to Chapter 2, 2.2.3.2). This suggests that these types of compound are not responsible for antiChE activity of these fractions; although may have been present at concentrations too low for detection; this would indicate compounds were very potent antiChEs. In addition, it cannot be excluded that such compounds are responsible for the antiChE activity of other fractions from all separation methods.

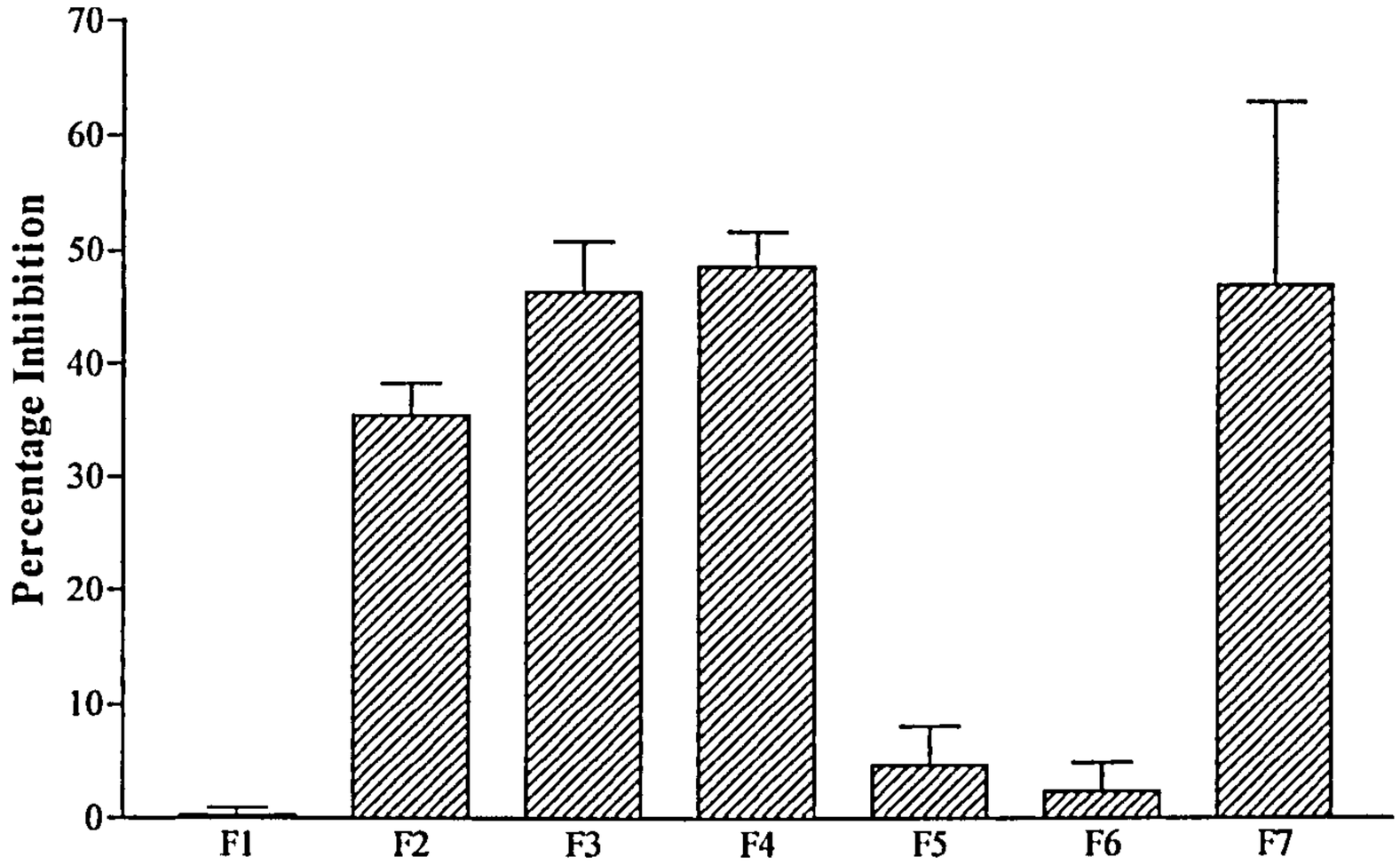


Figure 3.7. Inhibition of erythrocyte AChE by preparative thin layer chromatography fractions of *C. majalis* leaf (ethanol extract) (duplicate readings; n=3 ± SD).

Phenylalanine was present in F1 and F2 (determined by LC-MS analysis, refer to Chapter 2, 2.2.3.2), confirming any possible effect on AChE activity to be minimal. Unidentified glycoside compounds were present in F2 (refer to Chapter 2, 2.2.3.2), which also raises the possibility that these compounds were weak inhibitors of AChE. Compounds (yielding ions at *m/z* 277 and *m/z* 279) detected in both F3 and F4 confirm the results of the TLC analysis of these fractions, and may explain the similar antiChE activity of these fractions. The chemical nature of these compounds is yet to be identified, however F3 was negative for the presence of flavonoids (shown by HPLC analysis) (refer to Chapter 2, 2.2.3.2). The compounds (yielding ions at *m/z* 277 and *m/z* 279) were also detected in F6b and F7b (from FCC (b)) (refer to Chapter 2, 2.2.3.2), suggesting these compounds may also have contributed to antiChE activity

of these fractions. F7 contained a compound (yielding an ion at *m/z* 621), which was absent in F6 (refer to Chapter 2, 2.2.3.2); this compound may explain the antiChE activity of F7, but the structure of this compound remains to be elucidated.

3.2.4.6 Effect of Chlorophyll on Erythrocyte AChE Activity

The commercially obtained sample of chlorophyll used in this investigation was composed of several compounds (refer to Chapter 2, 2.2.5). These may include chlorophylls a and b, and perhaps chlorophyll derivatives and degradation products. Chlorophyll derivatives identified to date include pheophytins a and b, pheophorbide a and chlorophyllides a and b (Canjura and Schwartz, 1991; Harris *et al.*, 1995; van Breeman *et al.*, 1991). Chlorophylls a and b are two of the most abundant nitrogenous compounds in plants (Harborne and Baxter, 1993) and are composed of four nitrogen containing pyrrole rings chelated to a magnesium atom. It may be possible that degradation products of chlorophyll, containing nitrogen, may interact with the anionic active site of the AChE enzyme. However, the commercially obtained chlorophyll did not inhibit AChE activity when tested at 47.62µg/ml and at 95.24µg/ml. This suggests that chlorophyll may not be antiChE, and does not explain the activity of *C. majalis* leaf or fractions.

LC-MS analysis of the preparative TLC fractions confirmed that chlorophylls a and b (mw: 893.51 and 907.50 respectively) were absent in the active fractions (refer to Chapter 2, 2.2.3.2). However, it cannot be excluded that nitrogen-containing degradation products of chlorophyll present in the *C. majalis* extract, but absent in the reference chlorophyll solution, interfered with AChE activity, or were antiChE synergistically with other compounds.

3.3 Conclusion

Several herbs investigated for antiChE activity were inactive at the concentrations tested, including *Alisma orientalis* root, *Codonopsis pilulosa* root, *Gentiana* spp. (adulterated *Polygonum multiflorum* root), *Polygala tenuifolia* root, *Ziziphus jujuba* fruit and seed and *Ziziphus jujuba* var. *spinosa* seed (Table 3.1). The dried herbs of *Ziziphus jujuba*, *Polygala tenuifolia* and *Polygonum multiflorum* have previously been

investigated for their effects on AChE activity, with only *Polygala tenuifolia* showing significant AChE inhibition (40%) (Park *et al.*, 1996). However, the roots of *Polygala tenuifolia* and the seeds of *Z. jujuba* have not previously been investigated. The effect of *Polygonum multiflorum* root on AChE activity remains to be investigated, as the sample used for assessment of antiChE activity was adulterated, and found to be *Gentiana* spp. root (refer to Chapter 2, 2.2.1.1).

Results for extracts showing no AChE inhibition are not necessarily conclusive. Such extracts may have no effect on AChE inhibition; they may have antiChE activity but not at the physiological stage at which they were screened, they may contain compounds (thiol in particular) that interfere with the spectrophotometric test by reacting with DTNB, or they may contain cholinesterase constituents and consequently interfere with the assay. Cholinesterase activity has been established in a number of plants, particularly the Solanaceae, although their function is unknown (Gupta and Gupta, 1997; Schwartz *et al.*, 1964). The leaves, branch and stem of *Withania somnifera*, a member of the Solanaceae, have previously shown cholinesterase activity, but this activity was not detected in the roots (Gupta and Gupta, 1997). ACh (**1**) is reported to occur in some plant tissues, including *Phaseolus aureus* (the mung bean) (Fluck and Jaffe, 1974). The presence of ACh (**1**) in the extracts screened for antiChE activity may also interfere with the results. ACh (**1**) may compete with ATCh for the active site of AChE; consequently a reduction in released thiol would occur, lowering the concentration available for the reaction with DTNB.

From these investigations, it is apparent that several herbs contain AChE inhibiting compounds, which may explain their traditional use for memory and neurodegenerative disorders. Of the active herbs, some may yield known antiChE compounds, such as the monoterpenes (e.g. from *Centella asiatica* leaf), some may yield alkaloids previously unknown to inhibit AChE (e.g. from *Withania somnifera* root) and some may yield novel AChE inhibitors (e.g. from *Convallaria majalis* leaf). Studies on the reputed CNS effects of *Convallaria majalis* leaf to date are limited; therefore the potential for novel antiChE compounds, perhaps cardenolides, to be isolated from this herb would contribute to the understanding of structure-activity relationships for the AChE enzyme.

Although new compounds may inhibit AChE *in vitro*, a wide range of factors needs to be considered to assess therapeutic potential. One feature would be the

pharmaokinetic profile of a potential drug *in vivo*. Although a plant may have been used in traditional medicine for CNS disorders, investigations are required to establish if isolated compounds are able to cross the BBB and reach the CNS. It is reported that *in vivo* oral administration of *Salvia lavandulaefolia* essential oil to rats, inhibits AChE activity in striatum and hippocampus (Perry *et al.*, 2000b). This suggests that essential oil constituents have therapeutic potential in AD management, or may act as templates for the development of new drugs which are active in the CNS.

An appropriate adverse effect profile is also an important feature of a potential drug. The cardioactive effects of the cardenolides are well documented (David *et al.*, 1982; Grieve, 1984; Gupta and Chopra, 1987; Samuelsson, 1992). It is essential that a therapeutic dose selected to manage AD symptoms does not induce adverse effects such as cardiotoxicity. However, the potential antiChE activity of cardenolides, or other possible AChE inhibitors, may yield new therapeutic agents by acting as templates, and may minimise adverse effects associated with the original antiChE molecule.

CHAPTER 4

Assessment of Oestrogenic Activity of Plant Extracts, Essential Oils and Essential Oil Constituents

There are a variety of *in vitro* test systems available for the characterisation of the oestrogenic activity of various test substances, including the phyto-oestrogens. Assessment of oestrogenic activity may be achieved by conducting receptor binding assays, cell-proliferation assays, reporter gene assays and by *in vivo* investigations. It is important that a substance is assessed in various assay systems to evaluate the oestrogenic potency, so that as much information as possible regarding the oestrogenic potential of that substance can be obtained. In view of the current availability of very sensitive screens for oestrogenic activity (Diel *et al.*, 1999) the initial aim of this study was to investigate the oestrogenic activity of the plant extracts, essential oils and essential oil constituents using a recombinant yeast screen and the Ishikawa cell line. In the light of positive results obtained for some test substances, the study was extended to include both oestrogen (or estrogen) receptor (ER) binding and *in vivo* studies.

Initial screening of all plant extracts and essential oils was undertaken using an oestrogen-inducible yeast (*Saccharomyces cerevisiae*) screen expressing the human oestrogen receptor (hER) and containing expression plasmids carrying oestrogen-responsive sequences controlling the reporter gene lac-Z (encoding the enzyme β-galactosidase). The hER present in the yeast cell line is indistinguishable from hER expressed in human MCF-7 cells and affinity of 17β-oestradiol (E2) (**14**) for hER in the yeast cells is similar to that observed in the human MCF-7 cells (Metzger *et al.*, 1988). Upon binding of an active ligand to the hER, β-galactosidase is synthesised and metabolises the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG) to give chlorophenol red (CPR) (Figure 4.1). The amount of CPR formed is measured spectrophotometrically at 540nm to give a measure of the level of receptor binding.

Those test substances that were active in the yeast screen were also assessed for oestrogenic activity using the Ishikawa cell line, established by Nishida *et al.* (1985), which is derived from human endometrial carcinoma cells. This cell line is

unresponsive to oestrogens with respect to proliferation, but is sensitive to the specific stimulatory effect of oestrogens including phyto-oestrogens, on alkaline phosphatase activity; no other steroids including androgens, progestins, mineralocorticoids or glucocorticoids produce this effect (Littlefield *et al.*, 1990; Markiewicz *et al.*, 1993). Binding of an active ligand to the hER results in an increase in alkaline phosphatase activity. This activity is measured by addition of *p*-nitrophenol phosphate (*p*-NPP), a substrate for this enzyme. *p*-NPP is hydrolysed by alkaline phosphatase to *p*-nitrophenol (*p*-NP), a coloured product for which the optical density can be measured to give a measure of the receptor binding.

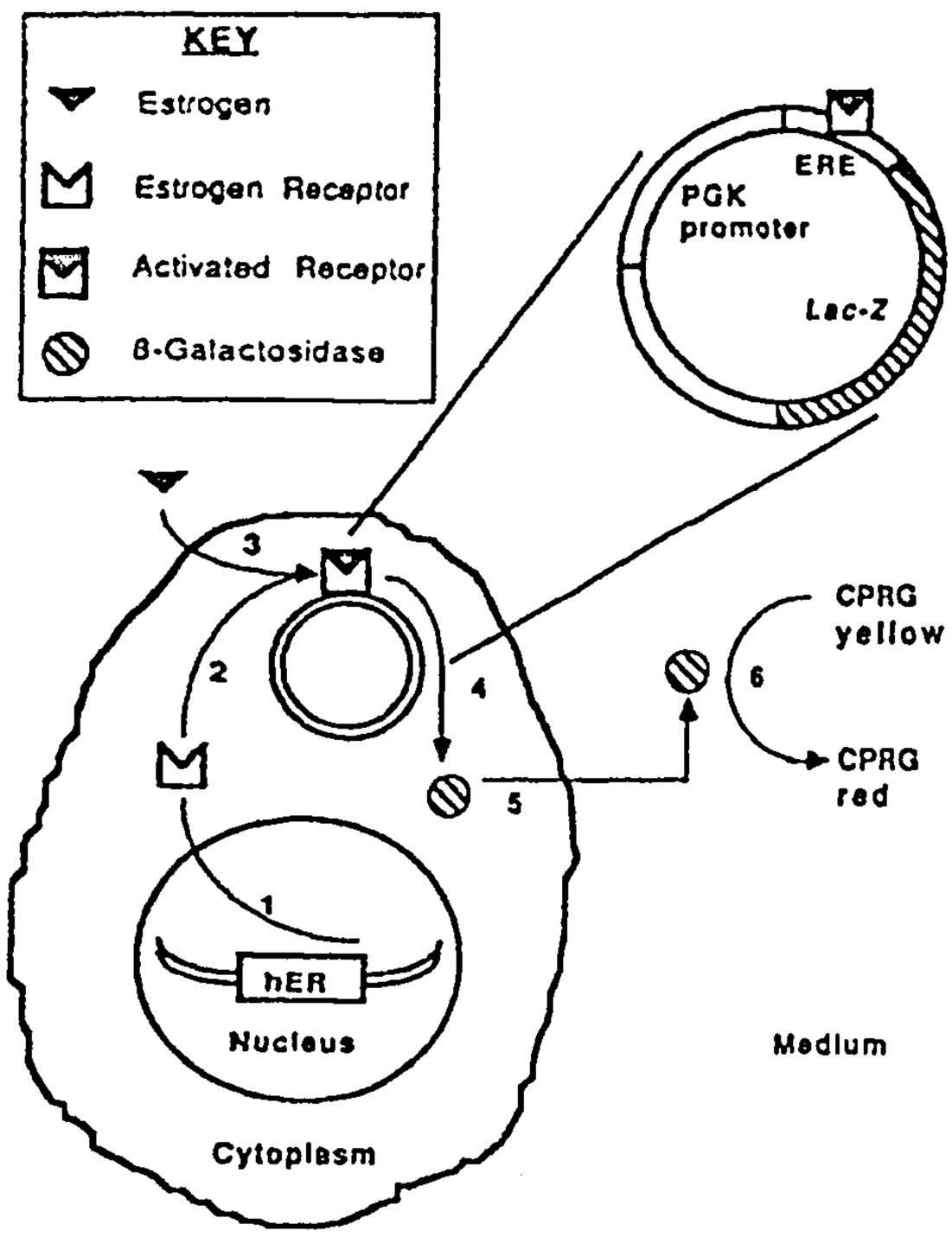


Figure 4.1. Schematic of the oestrogen-inducible expression system in yeast (Routledge and Sumpter, 1996). 1: The hER gene is integrated into the main genome and expressed in a form able to bind with the oestrogen (estrogen) response element (ERE). 2: Binding with ERE occurs within a hybrid promoter on the expression plasmid. 3: The hER is activated by binding of a ligand. 4: Expression of the reporter gene *Lac-Z* produces β-galactosidase. 5: β-galactosidase is secreted into the assay medium. 6: β-galactosidase metabolises CPRG (yellow) to give CPR (red).

Radioligand based Ishikawa cell receptor binding assays were performed to further investigate the receptor binding properties of the oil constituents. Binding assays were also conducted to determine the affinity of citral, eugenol (**84**), geraniol (**72**) and nerol (**75**) for isolated α-oestrogen receptor (ERα) and β-oestrogen receptor (ERβ). The isolated ERα and ERβ employed to assess ER binding were produced using recombinant baculovirus-infected insect cells (Brown and Sharp, 1990; Obourn *et al.*, 1993). Binding affinity of each of the compounds for the hER was determined by quantitation of ^{3}H-17β-oestradiol-receptor complexes.

Estimates of oestrogenic potency may differ, depending on the nature of the *in vivo* bioassay used (Milligan *et al.*, 1998). The oil constituents citral and geraniol (**72**) were therefore assessed for potential oestrogenic activity *in vivo* using two different assays. The first assay was based on the rapid vascular response of the uterine vasculature to oestrogenic stimulation (Arvidson, 1977; Milligan *et al.*, 1998), and the second was based on an uterotrophic assay (Odum *et al.*, 1997; Shelby *et al.*, 1996), which involves measurement of uterine weight following exposure to the test substances in the absence of endogenous oestrogen. In all investigations to assess oestrogenic activity, test substances were compared with the potency of the standard E2 (**14**), since this is the most commonly accepted positive control in both *in vitro* and *in vivo* assays (Blair *et al.*, 2000; Korenman, 1969; Miksicek, 1994).

The potential interactions (H-bond, electrostatic interactions and van der Waals forces) of the compounds citral (citral a (geranial) (**70**) and b (neral) (**71**)), eugenol (**84**), geraniol (**72**), and nerol (**75**) with the surrounding residues of the ligand binding domain (LBD) of the ERα were also investigated, to aid the interpretation of the results from the *in vitro* studies. This was achieved by using molecular graphics with the computer program HyperChem™.

4.1 Methods for Assessment of Oestrogenic Activity Using Reporter Gene Assays

4.1.1 Materials

Plant material, essential oils and essential oil constituents were obtained as described in Chapter 2, 2.1.1.1. Microtitre plates were obtained from Linbro, ICN Biochemicals, Thame, Oxon, England and sterile flasks were obtained from Nunclon®, Nunc Brand Products, Denmark. Unless otherwise stated, all other chemicals were purchased from Sigma, Fancy Road, Poole, Dorset, England.

4.1.2 Preparation of Assay Solutions for the Recombinant Yeast Screen

4.1.2.1 Preparation of Minimal Medium

Minimal medium was composed of 13.61g KH_2PO_4, 1.98g $(NH_4)_2SO_4$, 4.2g KOH pellets, 0.2g $MgSO_4$, 1ml $Fe_2(SO_4)_3$ solution (40mg/50ml H_2O), 50mg L-leucine, 50mg L-histidine, 50mg adenine, 20mg L-argenine-HCl, 20mg L-methionine, 30mg L-tyrosine, 30mg L-isoleucine, 30mg L-lysine-HCl, 25mg L-phenylalanine, 100mg L-glutamic acid, 150mg L-valine and 375mg L-serine, added to 1L double-distilled H_2O. Ingredients were dissolved on a heated stirrer then sterilised in an autoclave at 121°C for 10min and then stored at 4°C.

4.1.2.2 Preparation of Glucose Solution

A 20% w/v solution of D-(+)-glucose was prepared by adding 20g D-(+)-glucose to 80ml double-distilled H_2O. The solution was sterilised in an autoclave at 121°C for 10min and then stored at 4°C.

4.1.2.3 Preparation of L-Aspartic Acid Solution

A solution of 4mg/ml L-aspartic acid in double-distilled H_2O was sterilised in an autoclave at 121°C for 10min and then stored at 4°C.

4.1.2.4 Preparation of Vitamin Solution

8mg thiamine, 8mg pyridoxine, 8mg pantothenic acid, 40mg inositol and 20ml biotin solution (2mg/100ml H_2O) were added to 180ml double-distilled H_2O. The solution was sterilised by filtering through a 2.0μm pore size filter into a sterile glass vessel and then stored at 4°C.

4.1.2.5 Preparation of L-Threonine Solution

A solution of 24mg/ml L-threonine in double-distilled H_2O was sterilised in an autoclave at 121°C for 10min and then stored at 4°C.

4.1.2.6 Preparation of Copper (II) Sulphate Solution

A 20mM copper (II) sulphate solution was prepared by adding 160mg $CuSO_4$ to 50ml double-distilled H_2O. The solution was sterilised by filtering through a 2.0μm pore size filter into a sterile glass vessel and then stored at 4°C.

4.1.2.7 Preparation of Chlorophenol Red-β-D-Galactopyranoside Solution

A solution of 10mg/ml CPRG (Boehringer Mannheim, East Sussex, England) in double-distilled H_2O was sterilised by filtering through a 2.0μm pore size filter into a sterile glass vessel, then stored at 4°C.

4.1.2.8 Preparation of Culture Medium

Culture medium was prepared by adding 5ml glucose solution, 1.25ml L-aspartic acid solution, 0.5ml vitamin solution, 0.4ml L-threonine solution and 125μl copper (II) sulphate solution to 45ml minimal medium.

4.1.3 Method to Assess Oestrogenic Activity of Plant Extracts, Essential Oils and Oil Constituents Using a Recombinant Yeast Screen

The assays were carried out in 96-well plates as described by Routledge and Sumpter (1996) and were performed in a class II laminar air flow cabinet. 120µl concentrated yeast stock (a gift from Professor J. Sumpter, Brunel University, England) was added to 50ml freshly prepared culture medium with 0.5ml CPRG solution. 20µl aliquots of E2 (**14**) standard solutions diluted in EtOH (0.06nM - 1µM final assay concentration) and 20µl aliquots of plant extracts diluted in EtOH were added to wells and the EtOH evaporated (solutions of EtOH up to 40% do not denature β-galactosidase (Shifrin and Hunn, 1969)). 20µl aliquots of EtOH were added to wells as a control. 200µl of the yeast suspension in culture medium was added to each well. For volatile essential oils and oil constituents diluted in EtOH, 5µl aliquots were added to the sample wells and 5µl aliquots of EtOH were added to all other wells (including those containing the E2 (**14**) standard solutions) after the addition of 200µl yeast suspension to wells. Each volatile essential oil and each oil constituent were tested on separate plates to avoid cross contamination and false positive results. Plates were sealed and shaken for 2min on a titre plate shaker and incubated at 32°C. After 3 days (and shaking of plates for 2min each day), oestrogenic activity was determined from the metabolism of CPRG by monitoring the absorbance at 540nm using a MR 5000/7000 microtitre plate reader plus Reader Manager Software (Dynatech Laboratories Ltd., West Sussex, England).

4.1.4 Method to Assess Oestrogenic Activity of Essential Oils and Oil Constituents Using a Recombinant Yeast Screen, in Sealed Vessels

Freshly prepared culture medium containing concentrated yeast stock and CPRG was prepared as described above (4.1.2.8 and 4.1.3)), and 400µl aliquots were added to sterile glass vessels (1ml volume). Dilutions of E2 (**14**) and test substances (previously diluted in EtOH) were prepared by adding 10µl of the stock solution (of both E2 (**14**) and test substances) to 90µl of minimal medium. The assay concentration range for E2 (**14**) was 3.0×10^{-7}µM - 5.0×10^{-3}µM and for test substances, 0.3µM - 699.4µM. After shaking, 10µl aliquots of diluted E2 (**14**) and test substances were administered to the 400µl yeast suspension and the vessel contents were sealed

with the sterile vessel lid. Experiments were also conducted with the vessel lid not tightly closed, allowing gaseous exchange with the atmosphere. After shaking the vessel contents, samples were incubated at 32°C. After 3 days (and shaking of vessel contents each day), oestrogenic activity was determined by pipetting 200µl aliquots into the wells of a 96-well plate, and monitoring the absorbance at 540nm using a MR 5000/7000 microtitre plate reader plus Reader Manager Software (Dynatech Laboratories Ltd., West Sussex, England).

4.1.5 Method to Assess Metabolism of Compounds by Yeast Using GC-MS

To determine the extent of metabolism of 4 essential oil constituents (citral, eugenol (**84**), geraniol (**72**) and nerol (**75**)) by the yeast cells, each compound was incubated in the presence and absence of the oestrogen-responsive yeast, and the results compared using GC-MS analysis. Compounds diluted in EtOH (50µl) were added to 2ml culture medium containing yeast suspension and CPRG (as described above, 4.1.2.8 and 4.1.3) in 6-well plates to give the following assay concentrations: citral 445µM, eugenol (**84**) 495µM, geraniol (**72**) and nerol (**75**) 439µM. Duplicate plates containing test compounds but with culture medium only (no yeast suspension) were also prepared as controls. Each oil constituent was tested on separate plates to avoid cross contamination and false positive results. 1ml aliquots were removed from wells at the following time intervals: 0, 1, 3, 24, 48, 72hr and added to 1ml $(C_2H_5)_2O$ (BDH Supplies, Poole, England) in a sealed glass vessel. Following vigorous shaking the contents were allowed to equilibrate. After 1hr, the $(C_2H_5)_2O$ phase from each vessel was removed and stored in a sealed glass vessel at 4°C. The extracts were analysed using GC-MS (refer to Chapter 2, 2.1.10.2).

4.1.6 Method to Assess Anti-Oestrogenic Activity of Eugenol Using a Recombinant Yeast Screen

Eugenol (**84**) dilutions (in EtOH) were prepared and supplied to Dr Victoria Pocock, Endocrinology and Reproduction Research Group, King's College London, London, SE1 1UL, who conducted the yeast assay to investigate potential antagonistic activity of eugenol (**84**).

Freshly prepared culture medium containing concentrated yeast stock and CPRG was prepared as described above (4.1.2.8 and 4.1.3). 20µl aliquots of E2 (**14**) solution diluted in EtOH (0.78nM final assay concentration) were added to wells (in 96-well plates) and the EtOH evaporated. 20µl aliquots of hydroxytamoxifen (10^{-8}M - 10^{-5}M final assay concentration) were administered to wells as a positive control. 200µl of the yeast suspension in culture medium was added to each well, then 5µl aliquots of eugenol (**84**) (76.1µM - 609.0µM final assay concentration) diluted in EtOH were added to the sample wells and 5µl aliquots of EtOH were added to all other wells (including those wells containing the positive control). Plates were sealed and shaken for 2min on a titre plate shaker and incubated at 32°C. After 3 days (and shaking of plates for 2min each day), oestrogenic activity was determined from the metabolism of CPRG by monitoring the absorbance at 540nm using a MR 5000/7000 microtitre plate reader plus Reader Manager Software (Dynatech Laboratories Ltd., West Sussex, England). Absorbance readings were also obtained each day for 4 days thereafter, to determine the effect of potential eugenol (**84**) antagonism over time.

To determine if any apparent antagonistic effect of eugenol (**84**) was due to cytotoxic effects (as a decrease in absorbance could also be explained by cell death), yeast cells were counted in the presence of eugenol (**84**) at 76.1µM - 609.0µM, compared to the cell count in the presence of E2 (**14**) only (eugenol (**84**) absent). Cell counts were conducted by diluting 10µl assay suspension from each well with 200µl PBS, after the 3 day incubation period. Data are presented as the mean of 10 cell counts at each concentration.

4.1.7 Method for Assessment of Oestrogenic Activity in the Ishikawa Cell Assay

4.1.7.1 Maintenance and Passaging of Ishikawa Cells

Dr Jogen Kalita (Endocrinology and Reproduction Research Group, King's College London, London, SE1 1UL) is gratefully acknowledged for assistance with the maintenance and passaging of cells. Ishikawa Var-1 cells (a gift from Dr E. Gurpide, Mount Sinai School of Medicine, New York, USA) were maintained in culture medium (1:1 mixture of phenol red-free F12 HAM and DMEM), containing 1% amphotericin B solution, 1% sodium pyruvate (100mM) solution, 1% L-glutamine-penicillin-streptomycin solution (composed of 200mM L-glutamine, 10, 000U

penicillin and 10mg/0.9% NaCl streptomycin solution) and 5% foetal bovine serum (FBS). Cells were plated at $1.5x10^6$ cells/$75cm^2$ surface area in ventilated sterile flasks and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were passaged every 3 - 4 days by aspirating the culture media then adding 10ml HBSS (Ca^{2+} and Mg^{2+} free) to the flask. After shaking, 1ml trypsin/EDTA (0.25%) was added to digest protein surrounding the cells. Following incubation at 37°C for 3min - 5min, the flask contents containing the floating cells were centrifuged at 500g for 5min. The supernatant was aspirated and the cell pellet resuspended in 10ml culture media, and aliquots were transferred to sterile ventilated culture flasks ($1.5x10^6$ cells/$75cm^2$ surface area) with fresh culture media, and incubated at 37°C.

4.1.7.2 Assay Method

20µl aliquots of E2 (**14**) standards (0.02nM - 196.1nM), EtOH and test compounds were added to 96-well plates in the manner employed in the yeast screen assay (refer to 4.1.3), and allowed to evaporate to dryness. 24hr prior to each assay, the culture medium was replaced with assay medium (1:1 mixture of F12 HAM and DMEM), absent from endogenous steroids, and containing 1% amphotericin B solution, 1% sodium pyruvate (100mM) solution, 1% L-glutamine-penicillin-streptomycin solution (composed of 200mM L-glutamine, 10, 000U penicillin and 10mg streptomycin in 0.9% NaCl solution), 5% charcoal-stripped FBS and 0.6% D-(+)-glucose (45%) solution. Cells were then harvested with 1ml trypsin/EDTA (0.25%) and centrifuged (as described above, 4.1.7.1), then resuspended in assay medium. The cell suspension was diluted with assay medium to yield a cell density of 2.5×10^4 cells/100µl.

100µl aliquots of cell suspension were then administered to each well. For volatile essential oils and oil constituents diluted in EtOH, 2µl aliquots were added to the sample wells and 2µl aliquots of EtOH were added to all other wells (including those wells containing the E2 (**14**) standard solutions) after the addition of the cells. Each volatile essential oil and each oil constituent were tested on separate plates to avoid cross contamination and false positive results. The cells were incubated at a humidified atmosphere of 5% CO_2 and 95% air at 37°C for 72hr.

Following incubation, plates were inverted to remove growth medium, rinsed by immersion in phosphate buffered saline (PBS) and blotted dry. Plates were placed in

a freezer (-20°C) for 20min to rupture cell membranes then thawed at room temperature. 50µl of 5mM *p*-NPP was added to each well and the plates incubated at 37°C for 1hr. Oestrogenic activity was determined from the hydrolysis of *p*-NPP to *p*-NP by monitoring the absorbance at 405nm using a MR 5000/7000 microtitre plate reader plus Reader Manager Software (Dynatech Laboratories Ltd., West Sussex, England).

4.1.8 Data Analysis of Results Obtained Using the Recombinant Yeast Screen and the Ishikawa Cell Line

Data are presented as the mean (n=3-6 ± SD). Concentration dependent data were analysed using one-way ANOVA to determine the significance of the difference. Significance was regarded as $p<0.05$.

4.2 Methods for Oestrogen Receptor Binding Assays

4.2.1 Materials for Receptor Binding Assays

Essential oil constituents were obtained as described in Chapter 2, 2.1.1.1. Microtitre plates were obtained from Linbro, ICN Biochemicals, Thame, Oxon, England, [2, 4, 6, 7-^{3}H]-17β-oestradiol (84.0Ci.mmol^{-1}) was obtained from Amersham Life Science, Amersham, England and Optiphase Safe scintillation fluid was obtained from Wallac Scintillation Products and manufactured by Fisons Chemicals, Loughborough, Leicester, England. Unless otherwise stated, all other chemicals were purchased from Sigma, Fancy Road, Poole, Dorset, England.

4.2.2 Method for Ishikawa Cell Oestrogen Receptor Binding Assay

Ishikawa Var-1 cells were maintained and passaged as described previously (4.1.7.1). Cells were seeded in 6-well plates at a density of 100, 000 cells per well in culture medium and incubated at 37°C for 72hr, until cells were confluent. The culture medium was aspirated off and replaced with 2ml assay medium per well, to permit

uniform distribution of compounds in each well. 20µl aliquots of the test compounds (0.6mM - 5.9mM) and E2 (**14**) ($1.0x10^{-6}$mM - $1.0x10^{-3}$mM), diluted in EtOH, and 20µl EtOH alone as a control, were administered to wells. Each oil constituent was tested on a separate plate to avoid cross contamination and false positive results.
20µl aliquots of [2, 4, 6, 7-^{3}H]-l7β-oestradiol (15nM) diluted in assay medium were added to all wells, and plates were sealed and shaken for 2min on a titre plate shaker and incubated at 37°C for 1hr; plates were shaken at 20min intervals. The medium was aspirated off, and the plates were washed with 3 successive administrations of 2ml PBS to remove unbound ligand. 1.5ml EtOH was added to each well and plates were incubated at 25°C for 20min, to extract the ER-complexes. 1ml aliquots from each well were administered to scintillation vials with 4ml scintillation fluid. 20µl aliquots of [2, 4, 6, 7-^{3}H]-l7β-oestradiol diluted in assay medium were also vortex mixed with 1.5ml EtOH, and 1ml removed and added to 4ml scintillation fluid to obtain the total count. Vials were stoppered and vortex mixed prior to quantitation of the radioactivity, by recording CPM (10min for each measurement) using a β-scintillation counter (LS 6000IC, Beckman, UK).

4.2.3 Method for α- and β-Oestrogen Receptor Binding Assays

The potential oestrogen receptor binding activity of citral, geraniol (**75**), nerol (**75**) and eugenol (**84**) was studied using recombinant hERα and hERβ obtained from PanVera Corporation, Madison, USA, as described by Milligan *et al.* (2000). Test compounds or E2 (**14**) standard solutions were prepared in EtOH. Assay solutions were prepared as described in Table 4.1 and stored at 4°C. 20µl aliquots of the E2 (**14**) standards (0.18nM - 595.2nM final assay concentration) were administered to 1.5ml eppendorf tubes, and the EtOH was allowed to evaporate. Assay eppendorf tubes were placed on ice at 4°C for 30min prior to the addition of hot mix, ER solution and test compounds (201.0µM - 115930.1µM), as described in Table 4.2.

Table 4.1. Solutions for ERα and ERβ binding assays.

Solution	**Composition and Preparation**
Binding buffer	10mM Trizma preset crystals (pH 7.5), 10% glycerol, 2mM DDT and 1mg/ml BSA in distilled H_2O
ER solution	ERα or ERβ diluted in binding buffer (1:100)
ERα wash buffer	40mM Trizma preset crystals (pH 7.5), 100mM KCl, 1mM EDTA and 1mM EGTA in distilled H_2O
ERβ wash buffer	40mM Trizma preset crystals (pH 7.5) in distilled H_2O
Hydroxylapatite (HAP) slurry	10g HAP (Aldrich Chemical Company, New Road, Dorset, UK) added to 100ml Tris-EDTA, stirred and allowed to settle for 10min before the supernatant was aspirated off. This was repeated at least 6 times before allowing to equilibriate overnight at 4°C
Hot mix	15nM [2, 4, 6 , 7-^{3}H]-l7β-oestradiol diluted in binding buffer.
Tris-EDTA	50mM Trizma preset crystals (pH 7.5) and 1mM EDTA in distilled H_2O

Table 4.2. Administration of test compounds and assay solutions for ERα and ERβ binding assays.

Compound/ Control	**Hot Mix (µl)**	**ER Solution (µl)**	**Binding Buffer (µl)**	**Compound (in EtOH) (µl)**	**EtOH (µl)**
E2	100	10	0	20*	2**
Test compound	100	10	0	2**	0
Control 1 (C1)	100	0	10	-	2**
Control 2 (C2)	100	0	10	2**	0

* 20µl E2 solution added and EtOH evaporated before addition of other assay solutions.

** 2µl added after addition of all other assay solutions (i.e. added after hot mix, ER solution and binding buffer).

ER solutions were freshly prepared prior to each assay, and ERα and ERβ binding was assessed in different assays. Controls for each assay were conducted to determine the effect of the assay solutions (C1) and the effect of the test substances at all concentrations (C2), in the absence of the ER (Table 4.2). After vortex mixing, the assay tubes were incubated at 4°C overnight.

Following incubation, the free and bound ligand was separated. 100µl HAP slurry was added to all tubes, which were vortex mixed three times over a 15min incubation period, while on ice. Subsequently, 1ml wash buffer (ERα or ERβ wash buffer, depending on the ER used) was added to each tube; tubes were vortex mixed, centrifuged (2000g) for 15min and the supernatant discarded. After two further washes, the HAP pellet was extracted with two washes of 200µl EtOH, which were added to 4ml scintillation fluid in scintillation vials. The vials were stoppered and vortex mixed, and the radioactivity determined by recording CPM (10min for each measurement) using a β-scintillation counter (LS 6000IC, Beckman, UK).

4.2.4 Data Analysis of Results Obtained from the Receptor Binding Assays

Non-specific binding (B_{nsb}) was regarded as the binding of [^{3}H]-17β-oestradiol in the presence of the maximum concentration of unlabelled E2 (**14**) (10µM for the Ishikawa cell receptor binding assays; 595.2nM for the ERα and ERβ assays). This value was subtracted from the total amount of radioactivity bound in the presence of the test compound (B_{total}) to determine the amount of test compound bound. This was expressed as a percentage of the maximum radioactivity bound in the absence of the test compound (B_{max}), and the percentage inhibition of [^{3}H]-17β-oestradiol bound was calculated:

$$\text{Percentage inhibition} = 100 - \left[\frac{B_{total} - B_{nsb}}{B_{max} - B_{nsb}} \times 100 \right]$$

Data are presented as the mean (n=3-6 ± SD) and were analysed using one-way ANOVA to determine the significance of the difference. Significance was regarded as $p<0.05$.

4.3 Methods for *in vivo* Assessment of Oestrogenic Activity

4.3.1 Materials for *in vivo* Assays

Essential oil constituents were obtained as described in Chapter 2, 2.1.1.1. Unless otherwise stated, all other chemicals were purchased from Sigma, Fancy Road, Poole, Dorset, England.

4.3.2 *In vivo* Assay Methods

In vivo investigations were conducted by Dr Stuart Milligan, Endocrinology and Reproduction Research Group, King's College London, London, SE1 1UL.
Female Swiss albino mice (A. Tuck & Son Ltd., Battlesbridge, Essex, England) approximately 3 months of age and weighing 25g - 35g were maintained under constant conditions of lighting (lights on from 06:00hr to 18:00hr) and temperature (21 ± 1°C) and fed on a pelleted diet (Economy Rodent Maintenance, Essex, UK) *ad libitum*. All experiments were performed on ovariectomised animals. Ovariectomies were performed under tribromoethanol anaesthesia at least two weeks before the start of each experiment. All test compounds were administered transdermally, by applying 50µl - 100µl alcoholic solutions to the shaved backs of mice.

4.3.2.1 Acute Assay

In the acute assay of vascular responses, 100µl of alcoholic solutions of citral (1.9M), geraniol (**72**) (1.9M) and E2 (**14**) (0.3mM) or EtOH alone were administered transdermally. A quantitative index of the permeability of the uterine vasculature 4hr after subcutaneous (s.c.) injection of the test compound was obtained from the leakage of radiolabelled albumin from the circulation 3.5hr after the application of the test substance. 50µl of 0.5µCi [^{125}I]-labelled human serum albumin was injected into the jugular vein of mice anaesthetised with tribromoethanol; 30min later a blood sample was drawn from the suborbital canthal sinus with a heparinised capillary pipette and the animals killed by cervical dislocation. The blood sample was centrifuged to provide a 100µl plasma sample. The uteri and sample of thigh muscle were removed, briefly washed in saline and weighed. The radioactivity in the uterus, plasma sample

and muscle was determined. Previous studies on the uterus have indicated that the blood volume of the uterus is very much smaller than the albumin space (after a circulation time of 30min) and that the extravascular albumin space is the major determinant of the total tissue albumin space (Arvidson, 1977; Milligan and Mirembe, 1985). The tissue specific extravascular albumin volume (EAV) was expressed as 100 x ratio of the [^{125}I] cpm/mg of tissue to [^{125}I] cpm/µl plasma and used as an index of tissue vascular permeability.

4.3.2.2 Uterotrophic Assay

50µl of alcoholic solutions of citral (1.9M), geraniol (**72**) (1.9M) and E2 (**14**) (0.3mM) or EtOH alone were administered transdermally twice daily for 3 days. 12hr after the last administration the animals were killed, the uterine horns removed, blotted and weighed.

4.3.3 Analysis of Data Obtained from *in vivo* Investigations

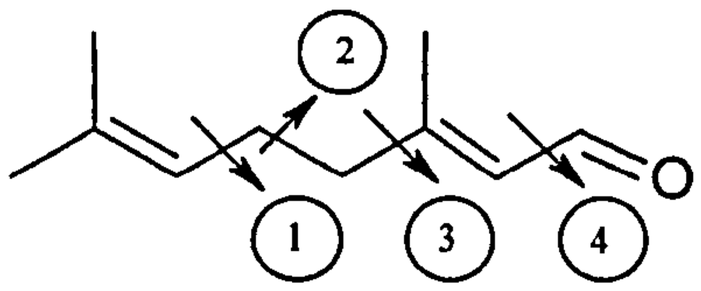
Data are presented as the mean (n=5 ± SEM) and were analysed using one-way ANOVA to determine the significance of the difference between the response following application of EtOH (control) and the response following the application of the test compound. Significance was regarded as $p<0.05$.

4.4 Method for Structure-Activity Assessment Using Molecular Graphics: Potential Interactions of Citral, Eugenol, Geraniol and Nerol with the α-Oestrogen Receptor

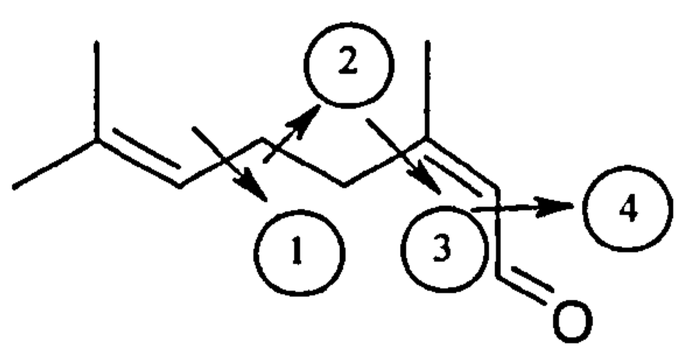
Assessment of the structure-activity relationship between ERα and receptor ligands (geranial (citral a) (**70**), neral (citral b) (**71**), eugenol (**84**), geraniol (**72**) and nerol (**75**)) was conducted using the computer program HyperChem™ (Autodesk Inc., Sausalito, California, USA).

Torsion angles (TOR) on the molecule under investigation were selected (Figure 4.2) prior to a conformational search performed for identification of low energy

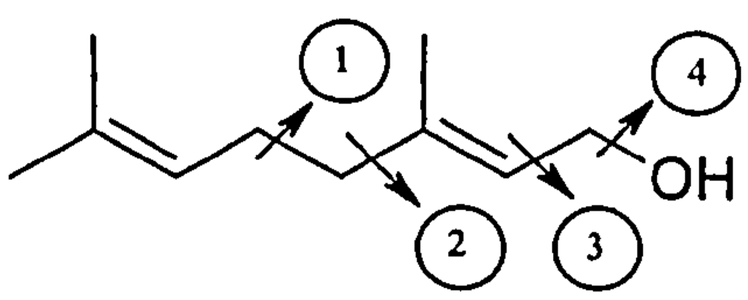
conformations. This method involves random variation of dihedral angles to generate new structures and subsequent energy minimising of each structure. Low energy conformations are used for further investigation, while high energy or duplicate structures are discarded. Random variation of acyclic bond dihedral angles uses rotation and for dihedral angles in a ring, angles are rotated by a motion of torsional flexing (Kolossváry *et al.*, 1993).



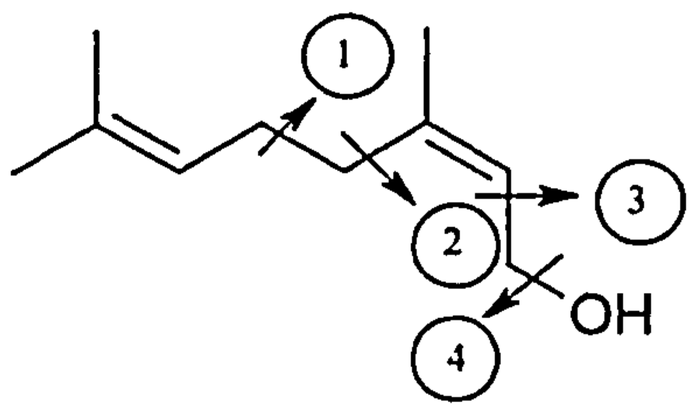
Geranial (citral a)



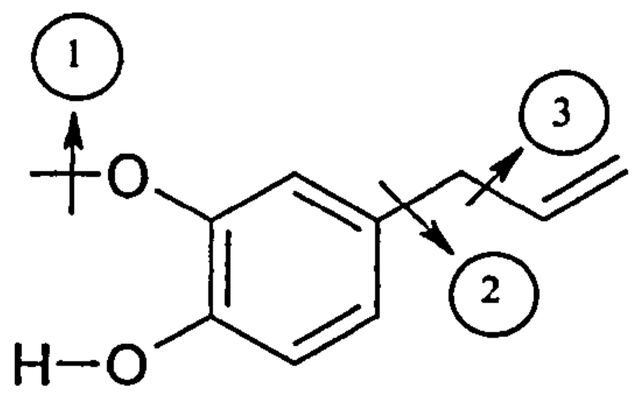
Neral (citral b)



Geraniol



Nerol



Eugenol

Figure 4.2. Torsion angles (TOR) on the molecules geranial (citral a), neral (citral b), geraniol, nerol and eugenol, selected prior to a conformational search to identify low energy conformations. 1: TOR 1, 2: TOR 2, 3: TOR 3, 4: TOR 4.

The search results generated gave the energy and torsion angles for each conformation of the molecule. Following inspection of the data, similar conformations of the molecule were grouped together (refer to 4.11, Tables 4.9 - 4.13). The conformation of lowest energy representative of each group was selected for further investigation. The conformers selected were compared with the structure of the known ER ligands

E2 (**14**) or raloxifene (RAL) (**95**), and one conformation of each molecule was selected. The selected conformation of each molecule was assessed for the ability to interact with the LBD of the ERα. This was achieved by manually introducing the molecule into the LBD of the ERα in such a way that it occupied a position similar to the bound ligands E2 (**14**) or RAL (**95**). For proposed ER agonist molecules (citral a (**70**), citral b (**71**), geraniol (**72**) and nerol (**75**)) the ERα structure in the presence of E2 (**14**) was selected for structure-activity investigations, and for the proposed ER antagonist molecule (eugenol (**84**)) the ERα structure in the presence of RAL (**95**) was selected.

Once the molecule being assessed was positioned in the LBD, then it's position and conformation were further adjusted, by refinement of the potential energy of the system. This was achieved by conducting a molecular mechanics optimisation using an MM+ force field and a conjugate gradient least squares refinement (Polak Ribiere) with termination criteria of 4000 maximum cycles or a potential energy gradient of 0.01kcal/[Å mol]. This process lowers the energy of the molecular system by adjusting its geometry, and therefore accounts for the position of the agonist/antagonist molecule within the LBD. The final torsion angles of each molecule positioned within the LBD were determined (Table 4.14).

An assessment was then made of the potential interactions (H-bond, electrostatic interactions and van der Waals forces) between the ligand and surrounding residues of LBD.

Once completed, the model complex was assessed using the computer program WebLab ViewerLite (MSI Inc., USA). The atomic van der Waals surfaces of the molecular system were displayed to check for steric complementarity. These investigations were also employed to demonstrate the interaction of E2 (**14**) and RAL (**95**) with ERα.

4.5 Results and Discussion: Assessment of Oestrogenic Activity of Plant Extracts

4.5.1 Assessment of Oestrogenic Activity of Plant Extracts, Using a Recombinant Yeast Screen

Aqueous and ethanolic extracts of each of the selected plants (*Alisma orientalis* root, *Apocynum lancifolium* leaf, *Centella asiatica* leaf, *Codonopsis pilulosa* root, *Convallaria majalis* leaf, *Gentiana* spp. root (adulterated *Polygonum multiflorum* root), *Melissa officinalis* leaf, *Polygala tenuifolia* root, *Rosmarinus officinalis* leaf (fresh and dried leaf), *Salvia miltiorrhiza* root, *Withania somnifera* root, *Ziziphus jujuba* seed and fruit, and *Ziziphus jujuba* var. *spinosa* seed) were assessed for oestrogenic activity over the concentration range $0.4g.L^{-1}$ - $3.5g.L^{-1}$, using the recombinant yeast screen.

Of the ethanolic and aqueous extracts of the plants screened, only the ethanolic extract of *Polygala tenuifolia* root demonstrated significant dose-dependent oestrogenic activity ($p<0.05$), EC_{50}: $1.3g.L^{-1}$ (Figure 4.3). All other extracts gave negative results. This suggests that oestrogenic compounds were absent in all other extracts, or may be present but were metabolised to inactive compounds, or may require metabolism by mammalian cells to give oestrogenic compounds. It is reported that some compounds that were oestrogenic *in vivo* (using a uterotrophic assay) were not oestrogenic in the yeast assay (Odum *et al.*, 1997), which perhaps reflects metabolic deficiencies in the yeast cells. It is also possible that the apparently inactive extracts may bind to the ER antagonistically. Further investigations are necessary, perhaps including antagonism of E2 (**14**) in the yeast assay or receptor binding studies, to identify potential antagonistic activity of the plant extracts.

Polygala tenuifolia root EtOH extract was considerably less potent than the positive control, E2 (**14**). The oestrogenic activity of the root extract may be due to constituents acting synergistically, but it cannot be excluded that a minor component of the root extract is a potent oestrogenic compound.

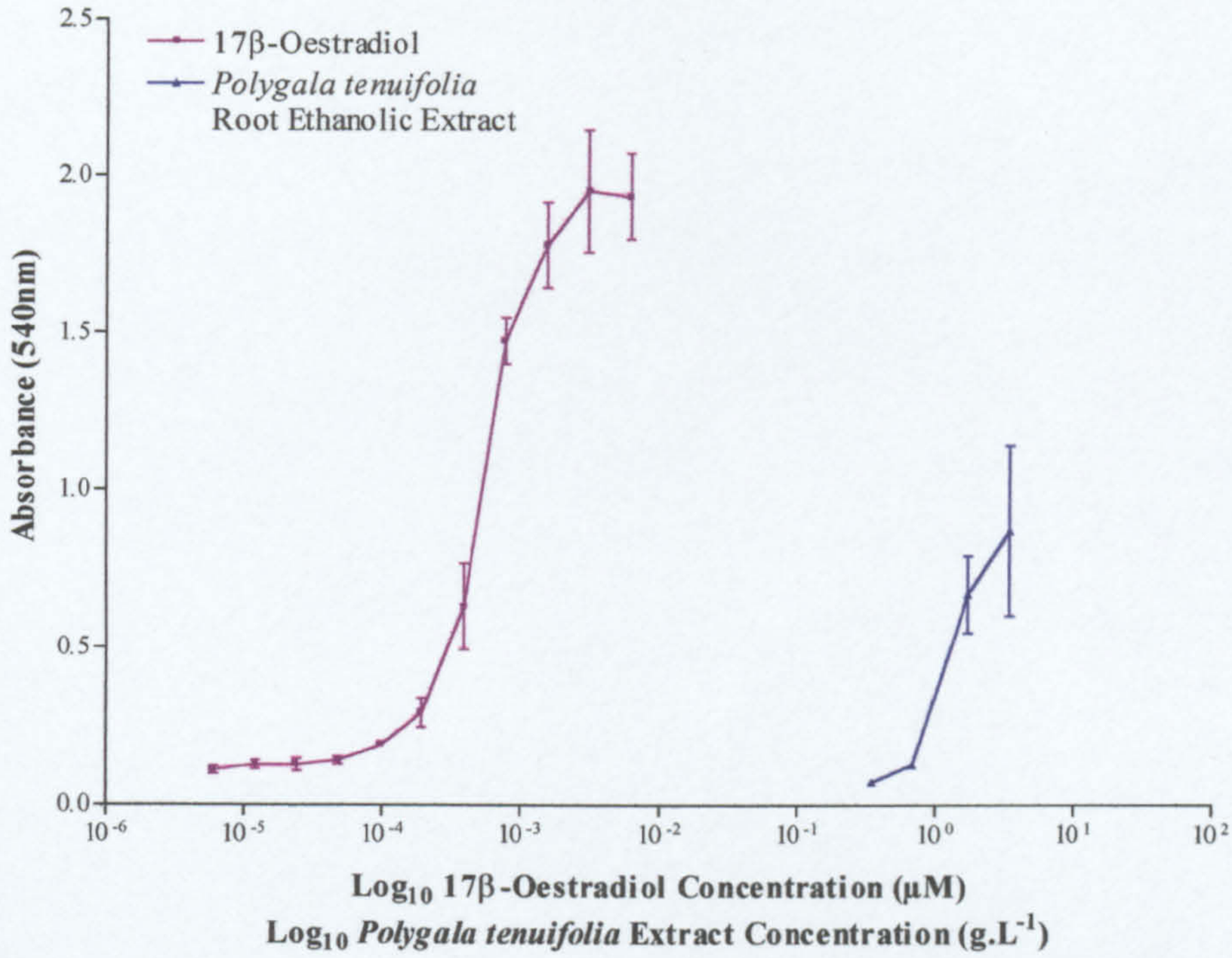
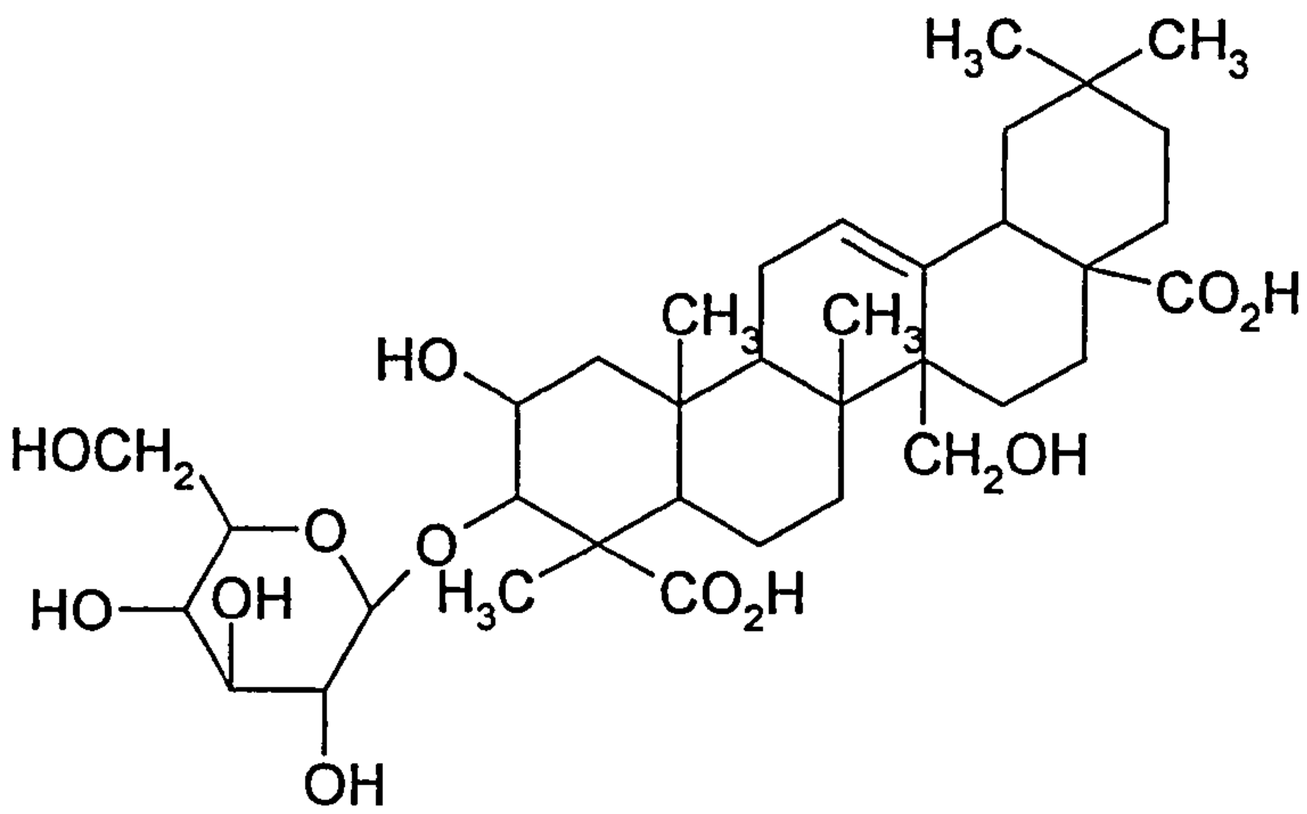


Figure 4.3. Oestrogenic activity of *Polygala tenuifolia* root ethanolic extract ($p<0.05$) and 17β-oestradiol ($p<0.001$), assessed by stimulation of β-galactosidase activity in genetically modified yeast cells (n=3-6 ± SD).

Polygala tenuifolia root has not previously been investigated for potential oestrogenic activity. As the aqueous extract of *Polygala tenuifolia* root did not demonstrate oestrogenic activity, it is apparent that the compounds responsible for oestrogenic activity were not highly polar. *Polygala tenuifolia* root is reported to contain oligosaccharides (e.g. tenuifolioses A - P), triterpene saponins (e.g. tenuifolin (**67**) and onjisaponins A - G) and xanthones (e.g. onjixanthones I and II) (Chang and But, 1986; Fujita *et al.*, 1992; Ikeya *et al.*, 1991a; Ikeya *et al.*, 1994; Miyase *et al.*, 1992; Sakuma and Shoji, 1981a; Sakuma and Shoji, 1981b; Tang and Eisenbrand, 1992).

The root is not known to contain constituents typically associated with oestrogenic activity, for example some isoflavones, coumestans and lignans, but that is not to say that such compounds were not present in the root and contributed to the observed oestrogenic activity.



Tenuifolin (**67**)

Xanthones are chemically related to flavonoids. It may therefore be possible that unidentified xanthones or related flavonoid compounds in the root extract contributed to the observed oestrogenic effects. The presence of steroidal saponins may also explain oestrogenic activity. Fractionation and isolation of compounds present in the EtOH extract is necessary to identify if typical or atypical phyto-oestrogens were responsible for the apparent oestrogenic activity but was not carried out in view of the weak activity, and because of priority given to other aspects of work reported in this thesis.

4.5.2 Assessment of Oestrogenic Activity of the Ethanolic Extract of *Polygala tenuifolia* Root, Using the Ishikawa Cell Line

To investigate the oestrogenic activity of *Polygala tenuifolia* root in mammalian cells, the EtOH extract was also investigated using the Ishikawa cell line. The extract showed significant dose-dependent oestrogenic activity ($p<0.05$) over the concentration range 27.3mgL^{-1} - 681.7mgL^{-1}, EC_{50}: 98.5mg.L^{-1} (Figure 4.4). As in the yeast screen, the crude EtOH extract was not as potent as E2 (**14**).

Phyto-oestrogens have consistently been reported to be less potent than the endogenous oestrogen E2 (**14**) (Coldham, *et al.*, 1997; Collins *et al.*, 1997; Markiewicz *et al.*, 1993; Milligan *et al.*, 1998; Milligan *et al.*, 1999; Murkies *et al.*, 1998), but that is not to say that phyto-oestrogens more potent than E2 (**14**) are absent from natural sources. To identify the potency, and perhaps synergy, of the potential

oestrogenic compounds in *P. tenuifolia* root, their isolation and evaluation in various assay systems to assess oestrogenic activity is required.

The Ishikawa cell line is reported to contain predominantly ERβ and the yeast cell line is reported to contain predominantly ERα (Dechering *et al*., 2000). The oestrogenic potency of *P. tenuifolia* root appeared to be greater in the Ishikawa cells (EC_{50}: 98.5mg.L^{-1}) than in the yeast cells (EC_{50}: 1.3g.L^{-1}, refer to 4.5.1), which leads to the suggestion that the root extract may have greater affinity for ERβ than ERα. Phyto-oestrogens are reported to have greater affinity for ERβ, which predominate in the prostate, bone and vascular tissue, whereas ERα predominates in the breast, uterus and ovary (Dechering *et al*., 2000; Kuiper *et al*., 1997; Mason, 2001). This suggests *P. tenuifolia* root extract may promote beneficial effects (e.g. in bone) but may not significantly induce adverse effects such as carcinogenesis (e.g. in breast tissue and ovaries).

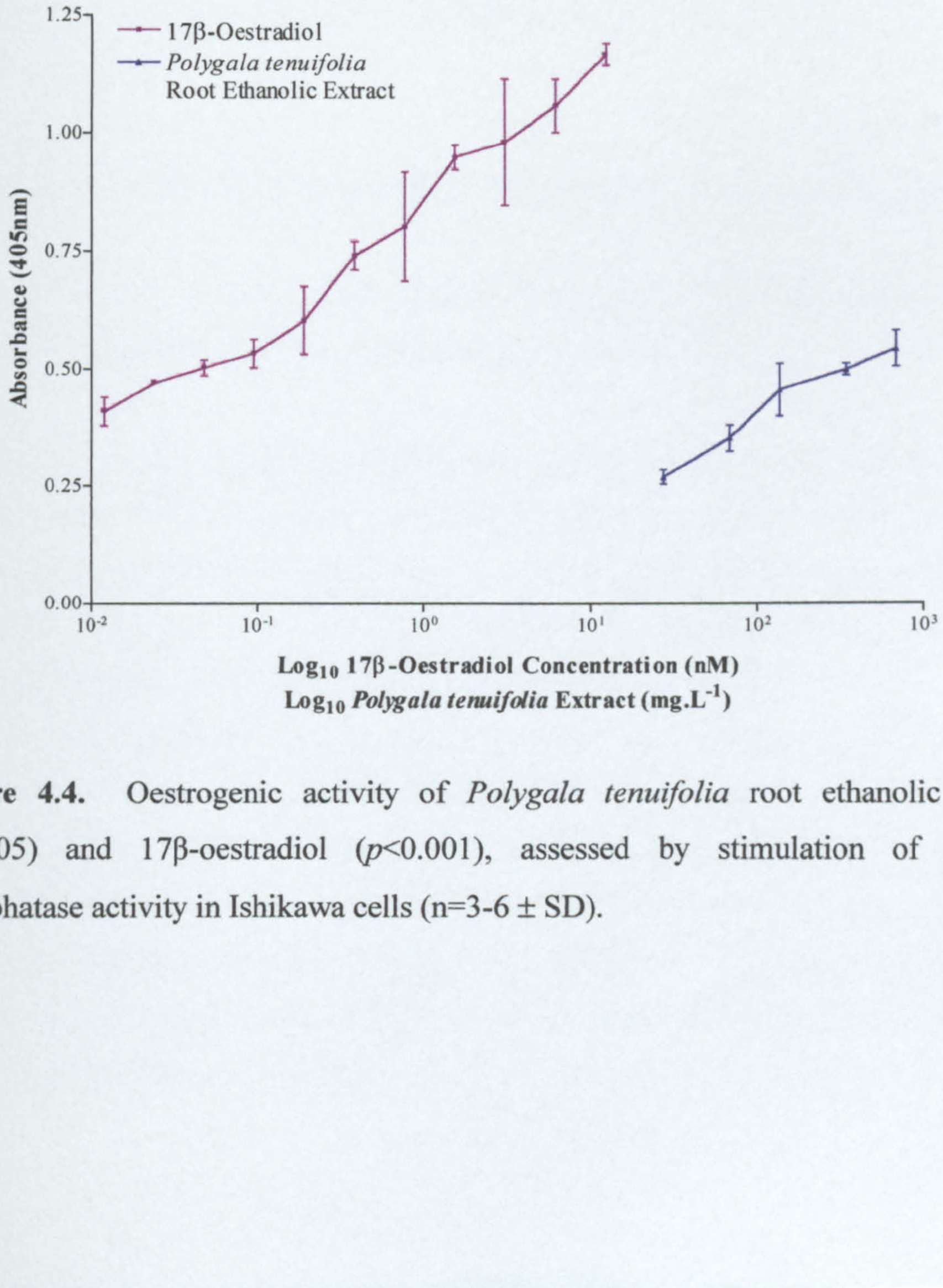


Figure 4.4. Oestrogenic activity of *Polygala tenuifolia* root ethanolic extract ($p<0.05$) and 17β-oestradiol ($p<0.001$), assessed by stimulation of alkaline phosphatase activity in Ishikawa cells (n=3-6 ± SD).

The localisation of ERβ in the neocortex, hippocampus and nucei of the basal forebrain (areas which were negative for ERα expression) indicates that ERβ may be responsible for mediating the beneficial effect of oestrogens on learning and memory (Fillit, 1994; Shughrue *et al.*, 1997; Wickelgren, 1997). Therefore, *P. tenuifolia* root extract may contain compounds that are selective for ERβ, with potential for beneficial effects on cognition and may also be important in the prevention of AD.

P. tenuifolia root EtOH extract was reported to strengthen the contractions and increase uterine muscle tone in pregnant and non-pregnant animals (Chang and But, 1986). ERβ has been located in the paraventricular and supraoptic nuclei, where ERβ has been suggested to regulate the expression of oxytocin, a hormone that stimulates uterine contraction (Alves *et al.*, 1998; Hrabovszky *et al.*, 1998). It may be feasible that the uterine contraction stimulated by *P. tenuifolia* root EtOH extract, reported by Chang and But (1986), may be explained by the root extract interacting with ERβ in the paraventricular and supraoptic nuclei, thus initiating oxytocin induced uterine contraction. Further investigations would be required to establish this.

4.6 Results and Discussion: Assessment of Oestrogenic Activity of Essential Oils

4.6.1 Assessment of Oestrogenic Activity of *Melissa officinalis* and *Rosmarinus officinalis* Essential Oils, Using a Recombinant Yeast Screen

Melissa officinalis essential oil demonstrated significant oestrogenic activity over the concentration range 0.1mg.L^{-1} - 124.4mg.L^{-1} (p<0.01), EC_{50}: 49.4mg.L^{-1}, but was cytotoxic at concentrations 622.0 mg.L^{-1} - 1243.9mg.L^{-1} (Figure 4.5). *Rosmarinus officinalis* essential oil did not demonstrate oestrogenic activity over this concentration range, nor was it cytotoxic (p>0.05) (Figure 4.5).

M. officinalis essential oil has not previously been reported to have potential oestrogenic effects. The major components of *M. officinalis* essential oil are monoterpenes (e.g. geranial (**70**)), but other components include sesquiterpenes (e.g. nerolidol (**83**)) and phenylpropanoids (e.g. eugenol (**84**)) (refer to Chapter 2, 2.2.7.1). The major components of the *M. officinalis* essential oil tested were citral (geranial (**70**) and neral (**71**)) and *trans*-caryophyllene (**82**), which comprised 39.6% of the total oil composition (refer to Chapter 2, 2.2.7.1). *R. officinalis* essential oil is reported to

contain 1, 8-cineole (**56**) (15-30%), camphor (**57**) (15-25%), α-pinene (**53**) (up to 25%) and other monoterpenes (e.g. borneol (**58**), bornyl acetate (**51**)) as the major constituents (Bisset, 1994; Trease and Evans, 1996). 1, 8-Cineole (**56**), camphor (**57**) borneol (**58**) and bornyl acetate (**51**) were not detected in the *M. officinalis* essential oil, and α-pinene (**53**) only comprised 1.3% of the total oil composition (refer to Chapter 2, 2.2.7.1).

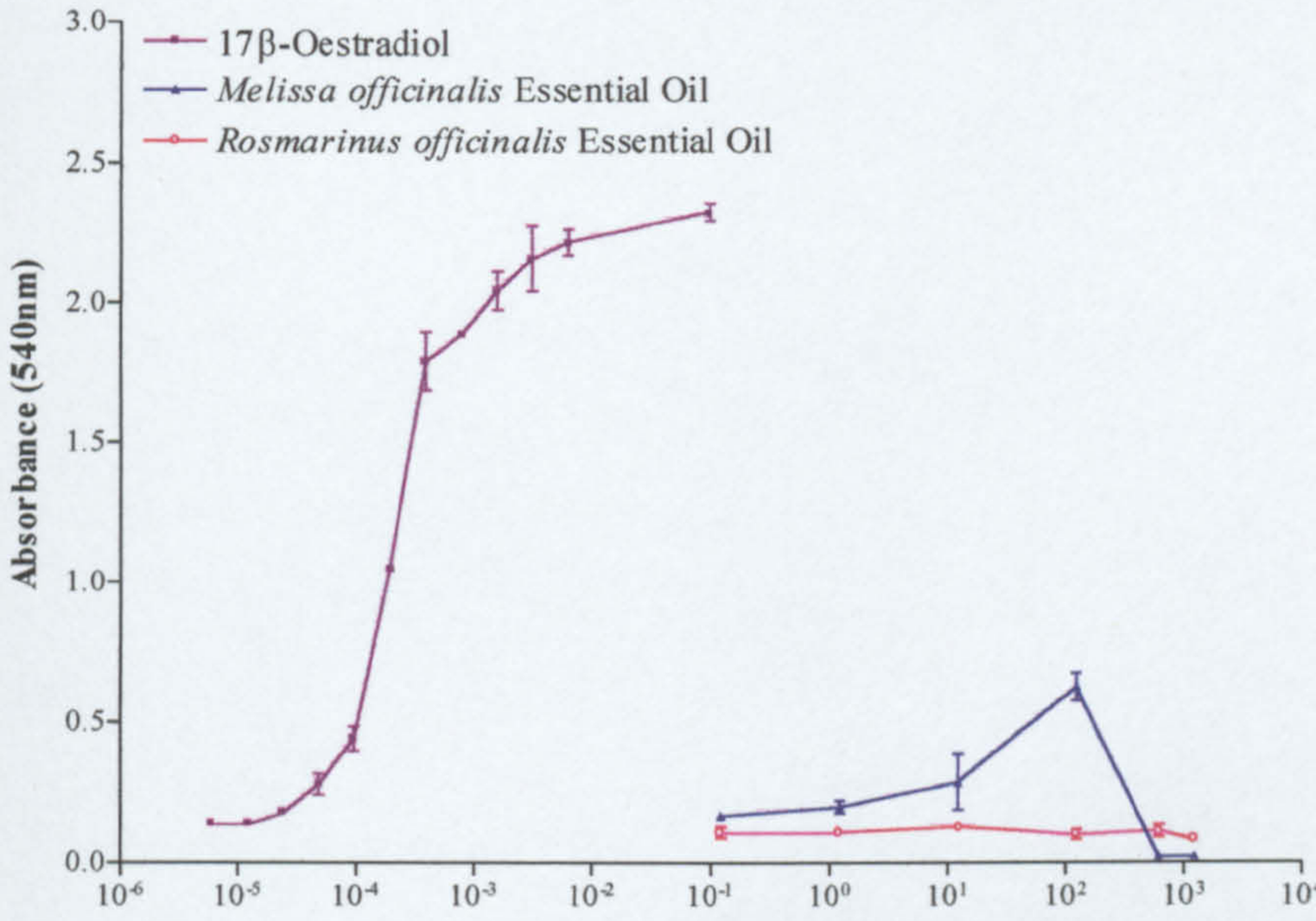


Figure 4.5. Oestrogenic activity of *Melissa officinalis* ($p<0.01$) and *Rosmarinus officinalis* ($p>0.05$) essential oils and 17β-oestradiol ($p<0.001$), assessed by stimulation of β-galactosidase activity in genetically modified yeast cells (n=3-6 ± SD).

The difference in composition between *M. officinalis* essential oil (which contains geranial (**70**), neral (**71**) and *trans*-caryophyllene (**82**) as the major components (refer to Chapter 2, 2.2.7.1)) and *R. officinalis* essential oil (which does not contain geranial (**70**), neral (**71**) and *trans*-caryophyllene (**82**) as major components (Bisset, 1994; Trease and Evans, 1996)), may explain the difference in the observed oestrogenic potencies of these oils. Further investigations were conducted to identify which

essential oil constituents may have been responsible for the oestrogenic activity of *M. officinalis* essential oil (refer to 4.7).

4.6.2 Assessment of Oestrogenic Activity of *Melissa officinalis* Phytol Extract and Phytol (Crude Extract), Using a Recombinant Yeast Screen

M. officinalis phytol extract ($p<0.01$) and phytol crude extract ($p<0.01$) also demonstrated significant oestrogenic activity in the yeast screen, over the concentration range 0.59mg.L^{-1} - 243.9mg.L^{-1} (Figure 4.6).

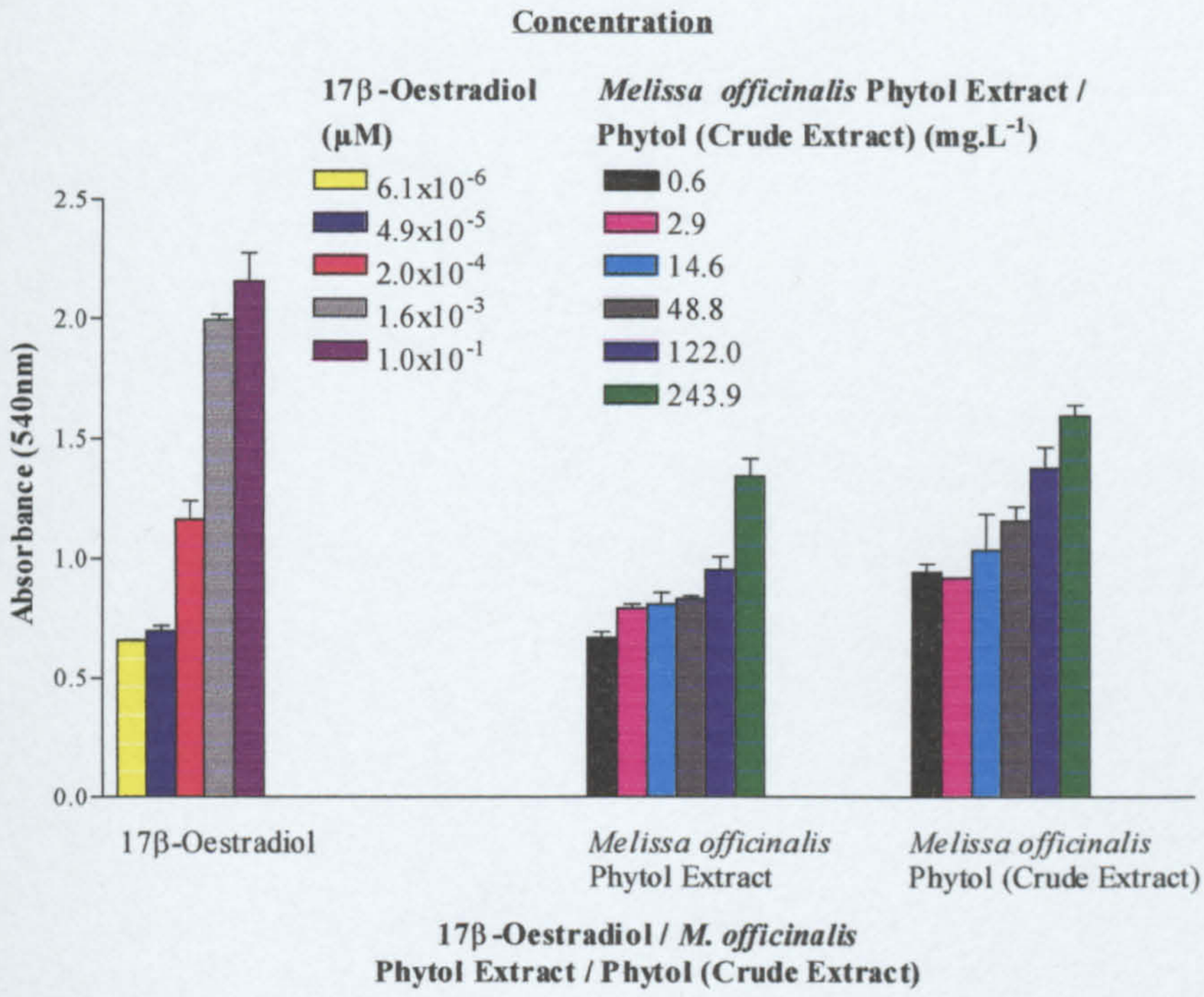


Figure 4.6. Oestrogenic activity of *Melissa officinalis* phytol extract ($p<0.01$), *Melissa officinalis* phytol crude extract ($p<0.01$) and 17β-oestradiol ($p<0.001$), assessed by stimulation of β-galactosidase activity in genetically modified yeast cells (n=3-6 ± SD).

M. officinalis phytol crude extract (EC_{50}: 85.8mg.L^{-1}) was more potent than *M. officinalis* phytol extract (EC_{50}: 138.5mg.L^{-1}). Neither the phytol extract nor the phytol crude extract were as potent as E2 (**14**), and both were less active than the *M. officinalis* essential oil (EC_{50}: 49.4mg.L^{-1}) (refer to 4.6.1).

The differences observed in oestrogenic potency between the *M. officinalis* essential oil, the phytol extract and the phytol crude extract may be explained by the differences in composition, as a result of the different extraction processes for each (refer to Chapter 2, 2.2.7.1). The phytols may also contain non-volatile compounds which may have been inactive or only weakly oestrogenic, which perhaps explains their weaker activity compared to the *M. officinalis* essential oil.

To explain the apparent activity of *M. officinalis* essential oil in the yeast screen, *M. officinalis* essential oil and some of the constituents identified in this oil (refer to Chapter 2, 2.2.7.1) were further investigated in other assays to gain further information regarding potential oestrogenic activity (refer to 4.6.3, 4.7, 4.8 and 4.9).

4.6.3 Assessment of Oestrogenic Activity of *Melissa officinalis* Essential Oil, Using the Ishikawa Cell Line

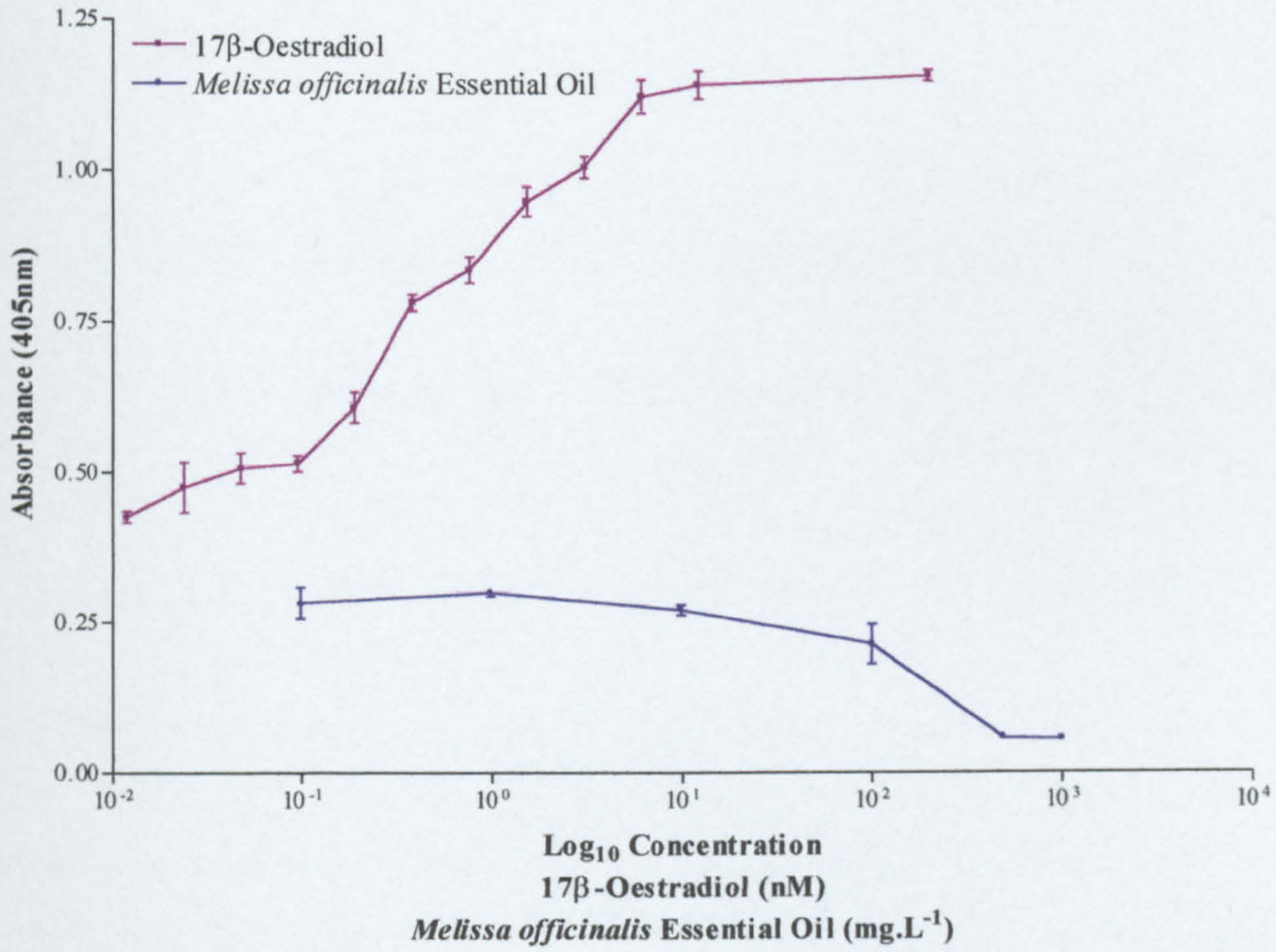


Figure 4.7. Oestrogenic activity of *Melissa officinalis* essential oil ($p>0.05$) and 17β-oestradiol ($p<0.001$), assessed by stimulation of alkaline phosphatase activity in Ishikawa cells (n=3-6 ± SD).

M. officinalis essential oil did not show significant oestrogenic activity in the mammalian Ishikawa cell line over the concentration range 0.1mg.L^{-1} - 993.3mg.L^{-1} (p>0.05) and was cytotoxic at concentrations ≥ 496.7mg.L^{-1} (Figure 4.7).

Although these results indicate that *M. officinalis* essential oil is not oestrogenic in the mammalian Ishikawa cells, other occurrences may explain the apparent inactivity. The essential oil constituents may interact with additional proteins, including enzymes in mammalian cells (which may be absent in the yeast cells), which may lower the concentration of an oestrogenic compound in the assay. The mammalian cells may have a different metabolic activity from the yeast cells, so may have metabolised compounds rendering them inactive, or an oestrogenic response may have occurred at the cytotoxic concentration, indicating inactivity.

4.7 Results and Discussion: Assessment of Oestrogenic Activity of Essential Oil Constituents

4.7.1 Assessment of Oestrogenic Activity of Some Monoterpenes Identified in *Melissa officinalis* Essential Oil, Using a Recombinant Yeast Screen

Some of the monoterpene constituents identified in *M. officinalis* essential oil (Şarer and Kökdil, 1991; Tittel *et al.*, 1982, and refer to Chapter 2, 2.2.7.1) were investigated for oestrogenic activity using the recombinant yeast screen, to identify which compounds may have been responsible for the observed oestrogenic activity of *M. officinalis* essential oil and phytol extracts.

This study suggests that some monoterpenes show evidence of weak oestrogenic activity. Of the monoterpenes tested, citral (p<0.001), citronellol (**69**) (p<0.001), geraniol (**72**) (p<0.001) and nerol (**75**) (p<0.001) showed significant oestrogenic activity in the yeast screen (Figures 4.8 and 4.9). These monoterpenes were identified in *M. officinalis* essential oil (refer to Chapter 2, 2.2.7.1) but are not reported to be major components in *R. officinalis* essential oil. Some of the major monoterpenes reported to occur in *R. officinalis* essential oil, 1, 8-cineole (**56**) (p>0.05) and camphor (**57**) (p>0.05) (Bisset, 1994; Trease and Evans, 1996), were not significantly oestrogenic (Figures 4.8 and 4.9). These results offer some explanation why the *M.*

officinalis essential oil demonstrated oestrogenic activity in the yeast screen and the *R. officinalis* essential oil did not (refer to 4.6.1).

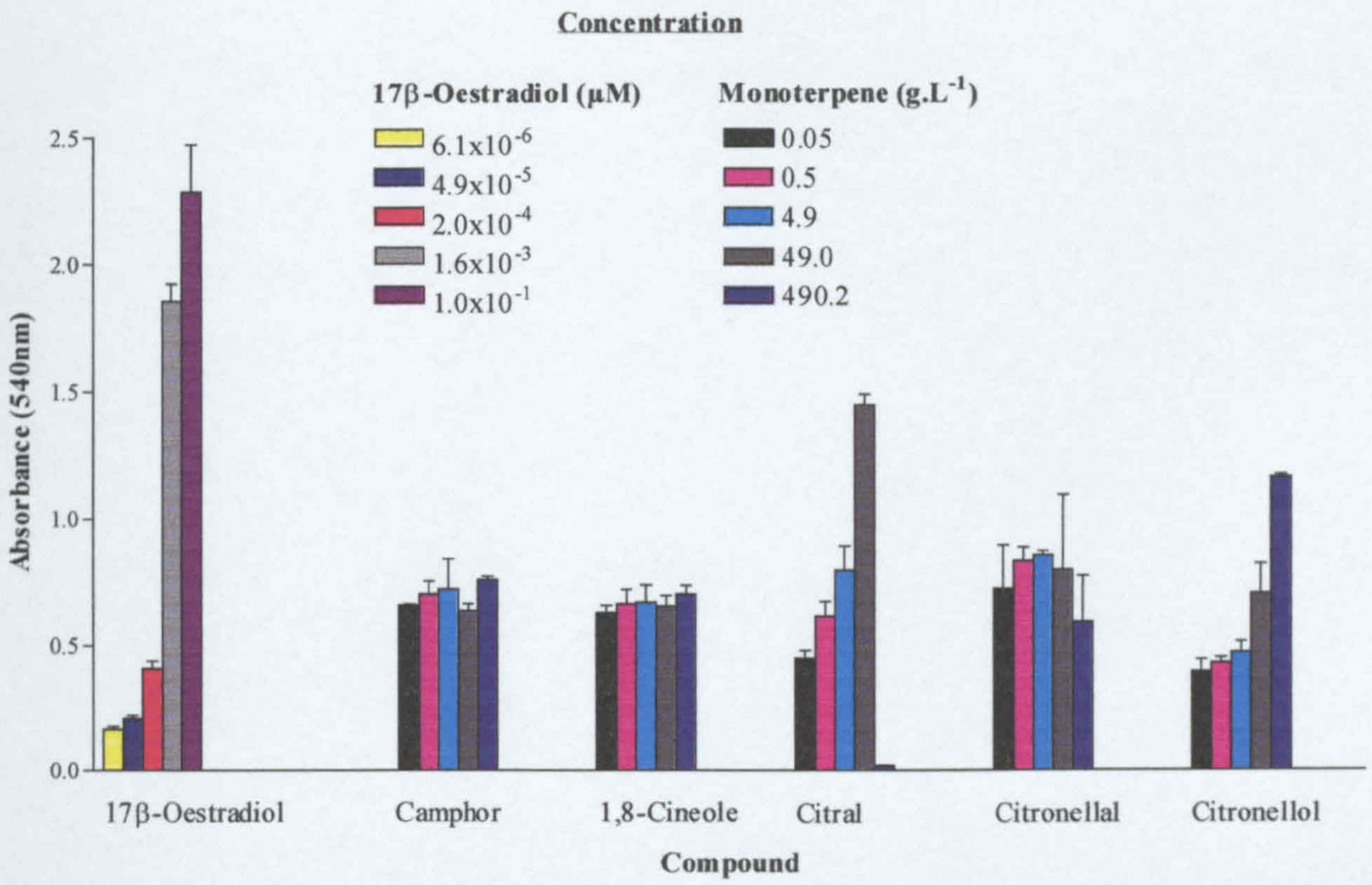
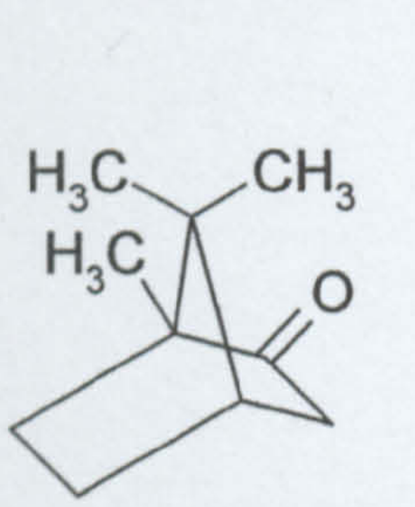
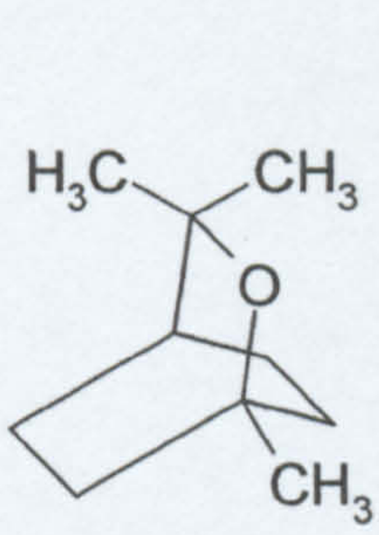


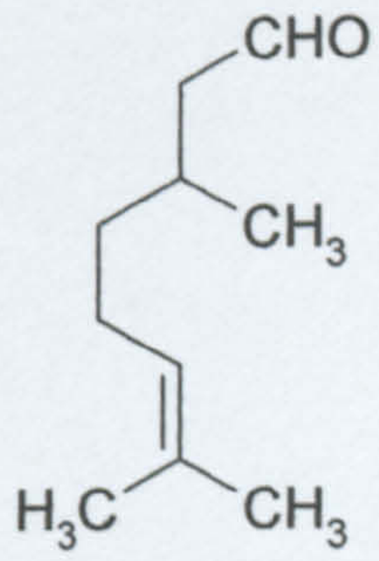
Figure 4.8. Oestrogenic activity of monoterpenes, camphor (p>0.05), 1, 8-cineole (p>0.05), citral (p<0.001), citronellal (p>0.05) and citronellol (p<0.001), and 17β-oestradiol (p<0.001), assessed by stimulation of β-galactosidase activity in genetically modified yeast cells (n=3-6 ± SD).



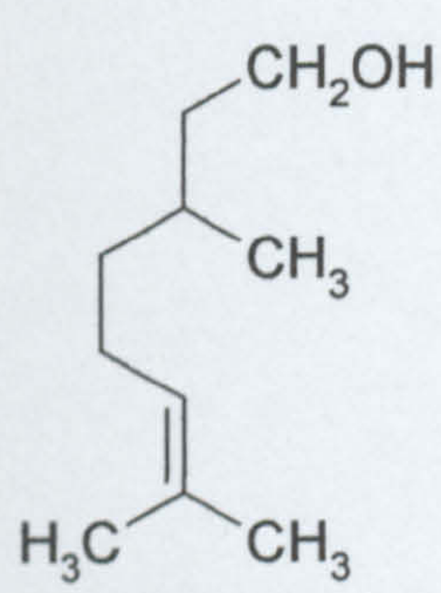
Camphor (**57**)



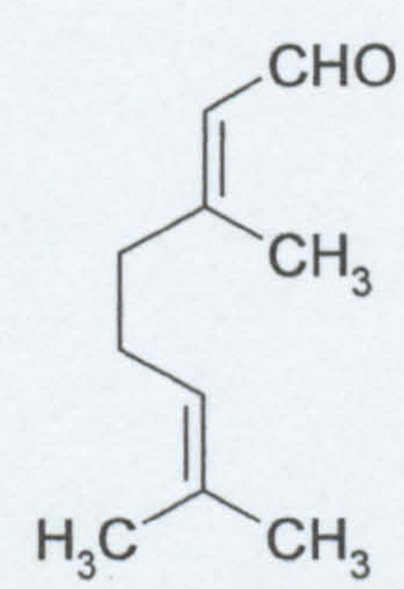
1, 8-Cineole (**56**)



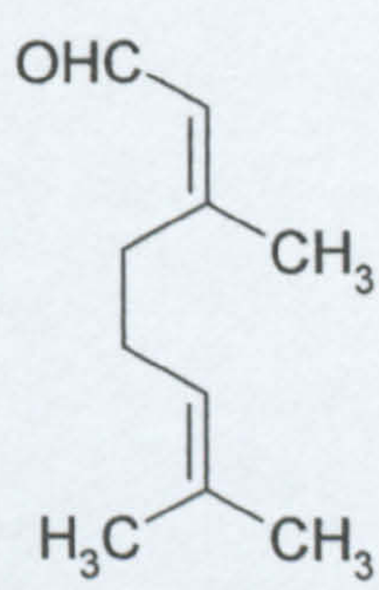
Citronellal (**68**)



Citronellol (**69**)



Geranial (Citral a) (**70**)



Neral (Citral b) (**71**)

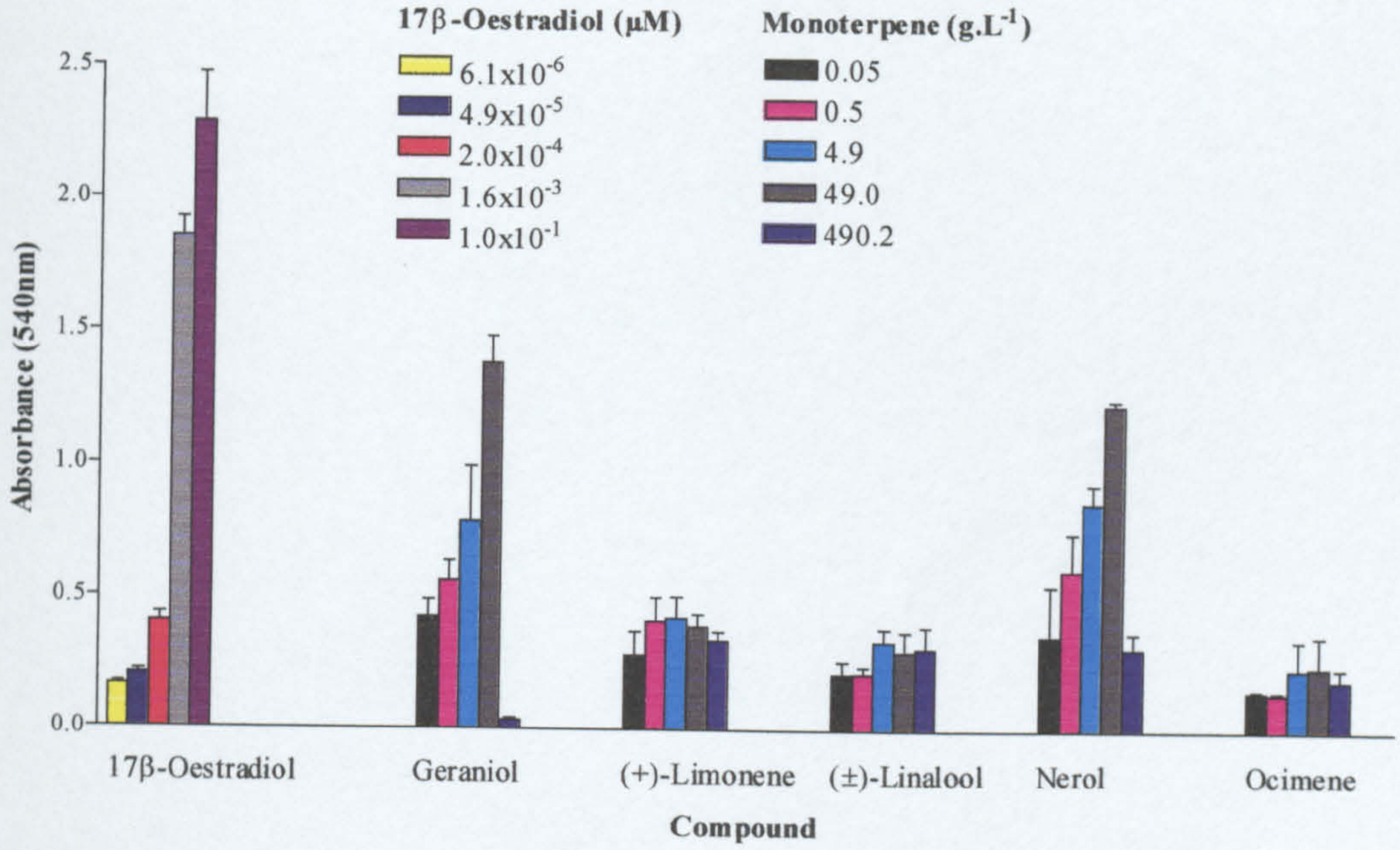
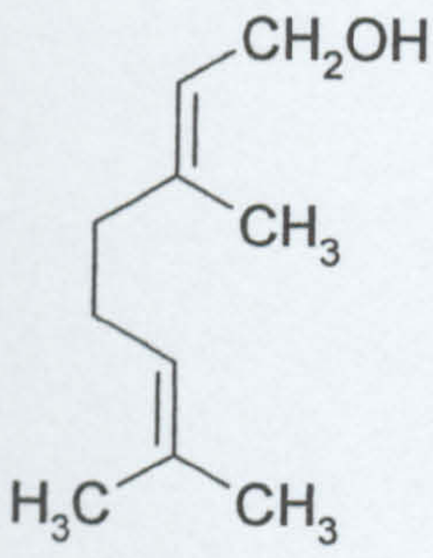
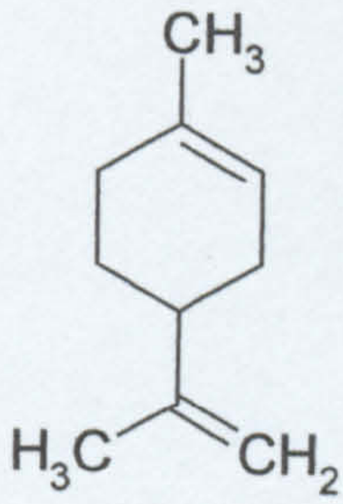


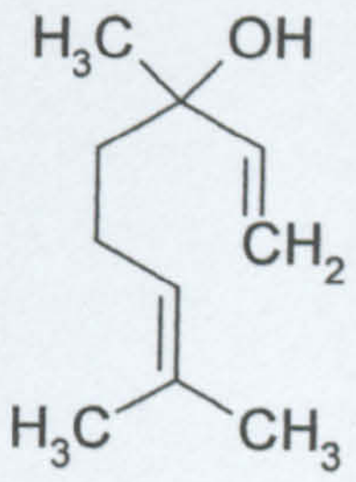
Figure 4.9. Oestrogenic activity of monoterpenes, geraniol (p<0.001), (+)-limonene (p>0.05), (±)-linalool (p>0.05), nerol (p<0.001) and ocimene (p>0.05), and 17β-oestradiol (p<0.001), assessed by stimulation of β-galactosidase activity in genetically modified yeast cells (n=3-6 ± SD).



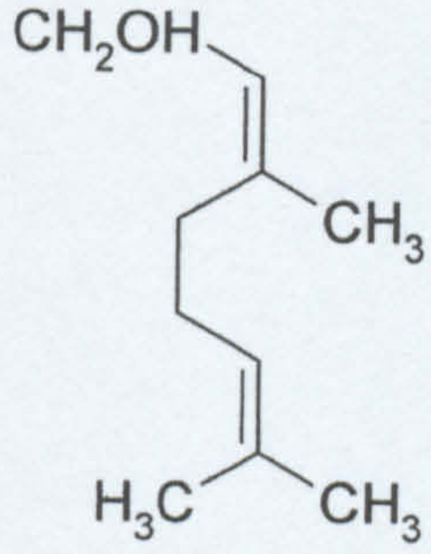
Geraniol (**72**)



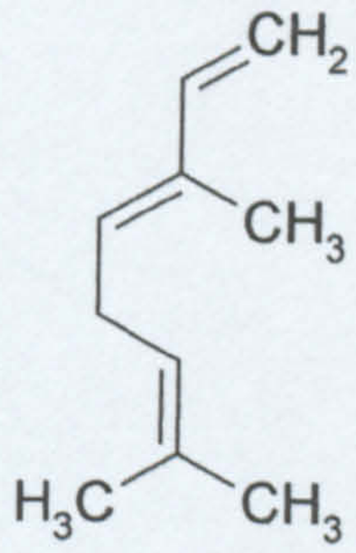
(+)-Limonene (**73**)



Linalool (**74**)



Nerol (**75**)



Ocimene (**76**)

Three monoterpenes, which demonstrated significant oestrogenic activity in preliminary studies (Figures 4.8 and 4.9), citral, geraniol (**72**) and nerol (**75**), were also assessed for oestrogenic activity using the recombinant yeast screen over the concentration range 1.652μM - $3.429x10^3$μM. Citral ($p<0.001$), geraniol (**72**) ($p<0.001$) and nerol (**75**) ($p<0.001$) showed significant oestrogenic activity, although citral and geraniol (**72**) displayed cytotoxicity at concentration $3.4x10^3$μM (Figure 4.10). The oestrogenic potencies (EC_{50} values) of E2 (**14**), citral, geraniol (**72**) and nerol (**75**) were $6.3x10^{-4}$μM, 343.4μM, 369.9μM and 1278.2μM respectively.

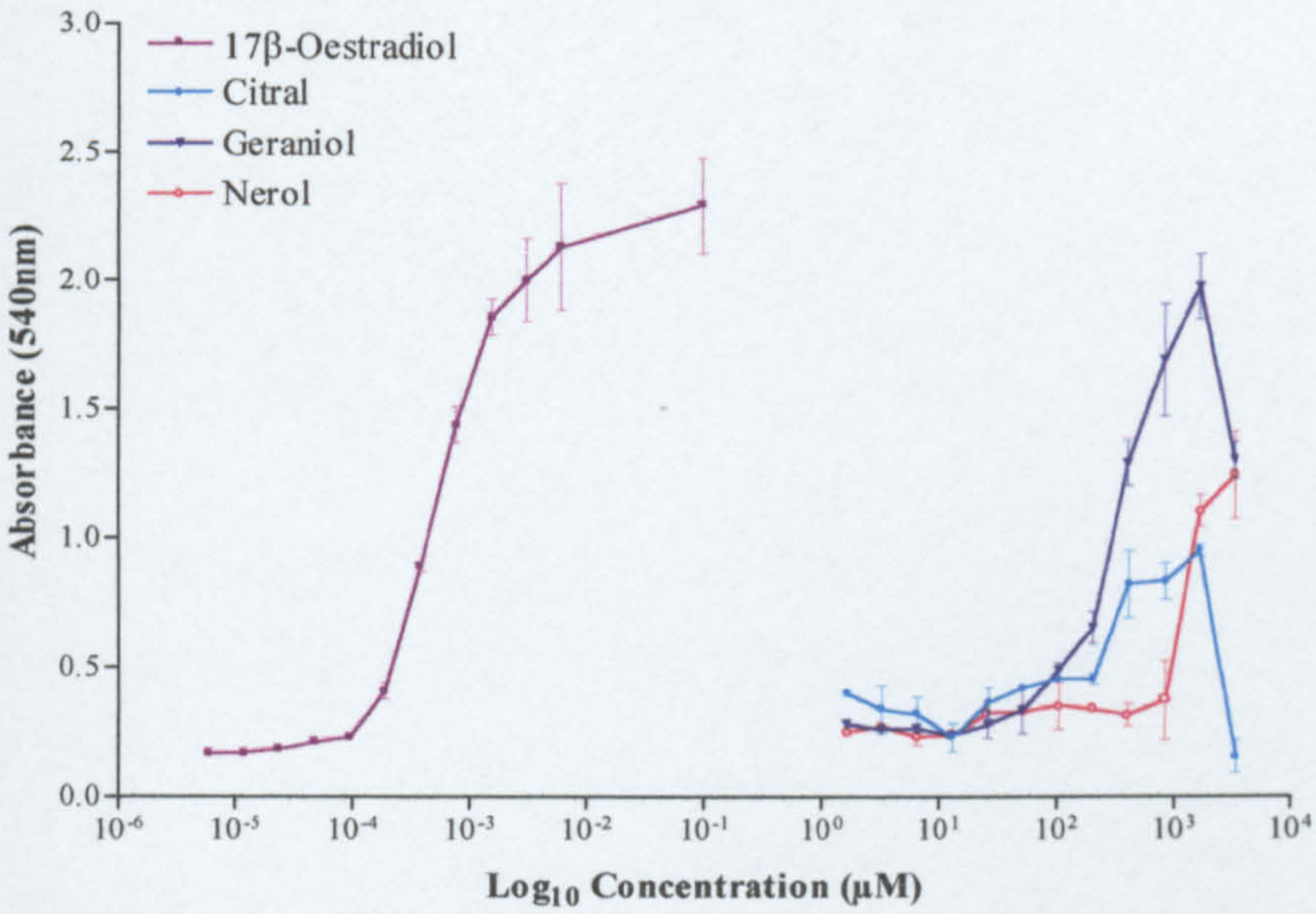


Figure 4.10. Oestrogenic activity of monoterpenes, citral ($p<0.001$), geraniol ($p<0.001$) and nerol ($p<0.001$), and 17β-oestradiol ($p<0.001$), assessed by stimulation of β-galactosidase activity in genetically modified yeast cells (n=3-6 ± SD).

The volatility of the these monoterpenes also resulted in oestrogenic responses being detected in the assay wells surrounding the test wells (which contained seeded assay medium alone), presumably reflecting the re-solution of the volatile compounds. This response decreased with increasing distance from the wells containing the monoterpene. For example, geraniol (**72**) treated wells (wells 6A, 6F, 8A, 8H, 10B, 10F, 12C and 12H) in a 96-well plate resulted in transfer of geraniol to the surrounding wells (Figure 4.11). A similar occurrence has been described for some

alkylphenols and some polychlorinated biphenyl (PCB) compounds, an effect which is described as "creeping" (Beresford *et al*., 2000).

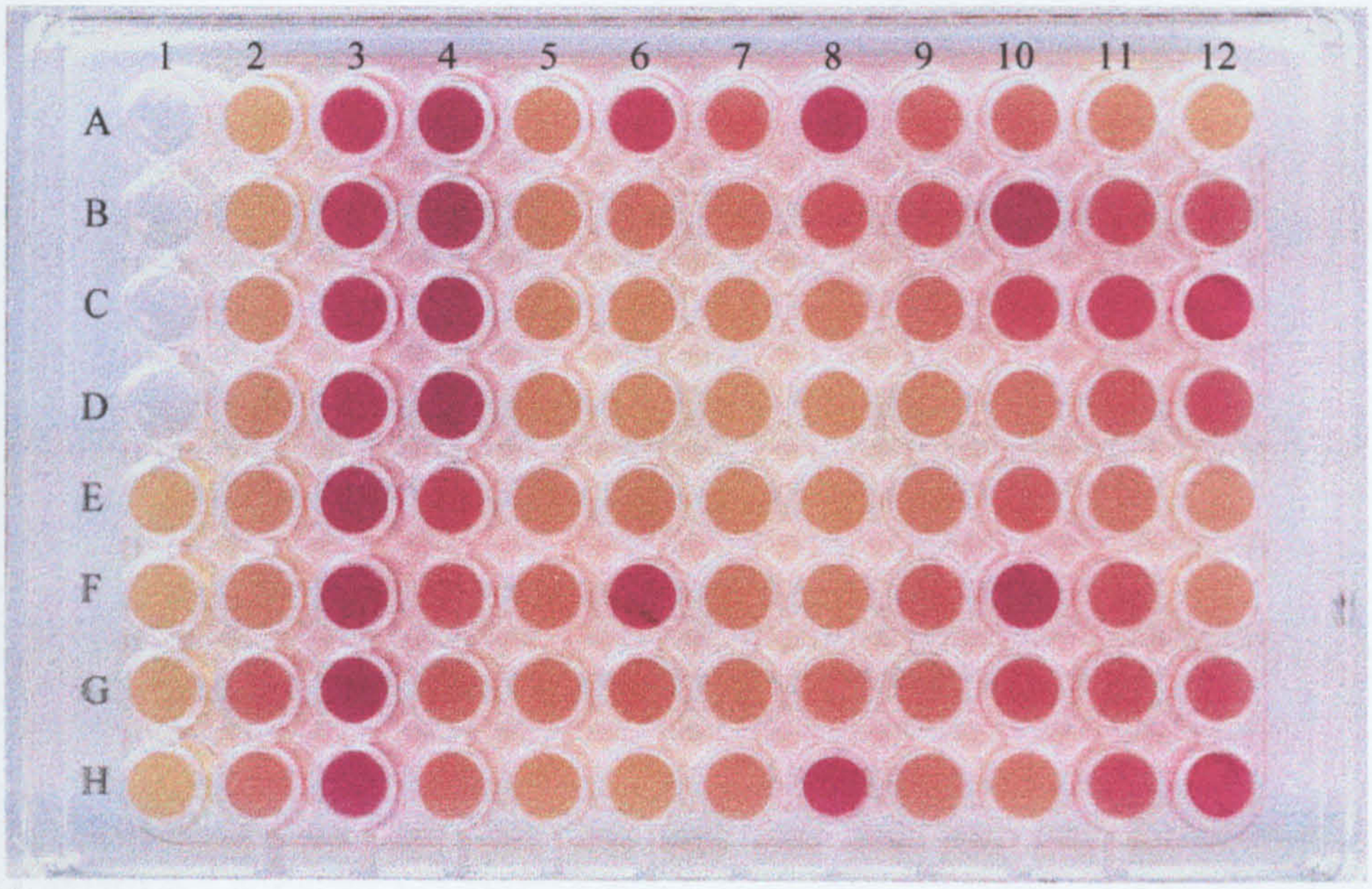


Figure 4.11. A 96-well plate containing a culture of genetically modified yeast cells treated with 17β-oestradiol (wells 1E - 4D) and geraniol (wells 6A, 6F, 8A, 8H, 10B, 10F, 12C and 12H).

The volatility of the monoterpenes citral, geraniol (**72**) and nerol (**75**) resulted in the loss of the compounds from the test wells and makes any interpretation of dose-response relationships very difficult. Therefore the final assay concentration may have been lower than the initial assay concentration, consequently the EC_{50} values may not reflect the oestrogenic potency of each compound, which may be more potent than indicated. This is additionally complicated by the metabolism of the compounds to other products (refer to 4.7.2 and Tables 4.3 - 4.5), perhaps with less oestrogenic activity. This may also have decreased the assay concentration of each monoterpene. Other considerations when interpreting the results are the presence of impurities in the assay. Impurities have been shown to give false positive results indicating oestrogenic activity (Harris *et al*., 1997). The percentage purities of the compounds citral, eugenol (**84**) geraniol (**70**) and nerol (**75**) were found to be >98%, >99%, >98% and 95% respectively, using GC-MS analysis (refer to Chapter 2, 2.2.7.2). Minor impurities were identified in some of these essential oil constituents including

alkanes, and in the nerol (**75**) sample analysed, geranial (**70**), geraniol (**72**) and neral (**71**) were also detected, and in the geraniol (**72**) sample analysed, geranial (**70**), neral (**71**) and nerol (**75**) were also detected (refer to Chapter 2, 2.2.7.2). Therefore the contribution of these impurities in the assay must also be considered, but their low concentrations indicate they are unlikely to have contributed significantly to the effects observed in the bioassays.

The precise structural requirements for oestrogenic activity are complex and are not yet fully understood. However it is generally accepted that a cyclic component (often phenolic) may be a structural requirement, as is a substituent (C3 hydroxyl for E2 (**14**)) acting primarily as a hydrogen bond donor in the LBD of hER (Blair *et al.*, 2000; Dodge, 1998). Therefore, the acyclic monoterpenes (citral a (geranial) (**70**), citral b (neral) (**71**), citronellol (**69**), geraniol (**72**) and nerol (**75**)) are novel as potential hER ligands. Citral, citronellol (**69**), geraniol (**72**) and nerol (**75**) have aldehyde or hydroxyl substituents that may interact with the hER (refer to 4.11 for further analysis). The monoterpenes that were not significantly oestrogenic may have lacked the structural features required for hER binding. For example, limonene (**73**) and ocimene (**76**) are hydrocarbons, which lack substituents perhaps required to act as a hydrogen bond donor in the LBD of hER.

It must also be considered that, although some monoterpenes did not demonstrate significant oestrogenic activity in the yeast screen, it cannot be concluded that such compounds had no affinity for the hER. A hER antagonist may have bound to the hER in the yeast screen, but would not have initiated the oestrogenic response resulting in an increase in β-galactosidase activity. Further investigations are required including ER binding studies, to identify which of the monoterpenes are ligands for the hER and are perhaps hER antagonists.

4.7.1.1 Assessment of Oestrogenic Activity of Geraniol in a Sealed Environment, Using a Recombinant Yeast Screen

Geraniol (**72**) (2.7μM - 690.3μM) was assessed for oestrogenic activity using the recombinant yeast screen, in sealed glass vessels (with lids tightly closed) and compared with E2 (**14**) ($1.2x10^{-7}$μM - $5.0x10^{-3}$μM). However, yeast cell death occurred at all concentrations in the absence and in the presence of both compounds.

This indicates that cell death was independent of the presence of other compounds and may have occurred due to the restricted culture conditions (e.g. insufficient O_2).

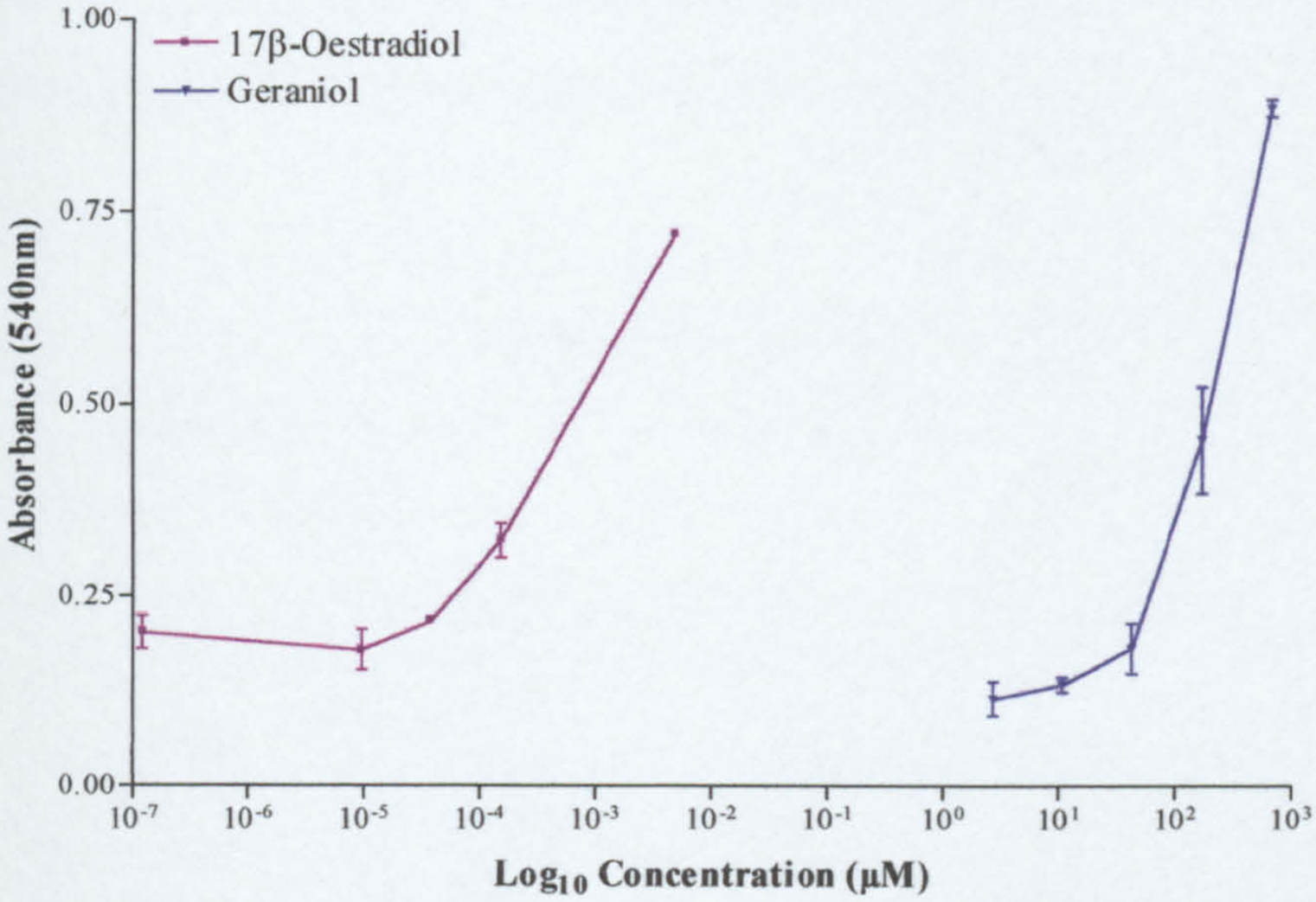


Figure 4.12. Oestrogenic activity of geraniol (p<0.001) and 17β-oestradiol (p<0.001), assessed by stimulation of β-galactosidase activity in genetically modified yeast cells, in a restricted environment (n=3-6 ± SD).

To investigate this, the assay was repeated in the sealed glass vessels but with the lids not closed tightly, allowing gaseous exchange with the atmosphere. Yeast cell death did not occur, and an oestrogenic response was observed with E2 (**14**) (p<0.001) and with geraniol (**72**) (p<0.001) (Figure 4.12). These results indicate that the optimum gaseous supply to the yeast cells also permits loss of volatile compounds (e.g. geraniol (**72**)) from the culture medium, which makes interpretation of dose-dependent relationships difficult.

4.7.2 Assessment of the Metabolism of Citral, Geraniol and Nerol, in a Recombinant Yeast Screen

The products of metabolism following treatment of the yeast culture with the essential oil constituents was determined over 72hr to investigate the role of metabolism in biological activity; analysis was by GC-MS (for method, refer to Chapter 2, 2.1.10.2).

Citral (geranial (**70**) and neral (**71**)) concentration decreased with time in the presence of yeast, and was not detected after 48hr (Table 4.3).

Table 4.3. Assessment of metabolism of citral (geranial and neral) by yeast. Compounds detected in the presence and absence (*x*) of yeast. Peak area determined by ITD.

	Peak Area for Compound Detected					
Time (h)	**Citronellol**	**Geranial**	**Geraniol**	**Neral**	**Nerol**	**Phenylethyl Alcohol**
0	0 (0)	1.55 (2.35)	0 (0)	1.70 (2.35)	0 (0)	0 (0)
1	0 (0)	1.25 (2.10)	0.02 (0)	1.65 (2.1)	0 (0)	0 (0)
3	0.04 (0)	0.65 (2.05)	0.08 (0)	0.80 (2.05)	0.04 (0)	0.03 (0)
24	0.35 (0)	0.04 (1.10)	1.45 (0)	0.25 (1.10)	0.30 (0)	0.10 (0)
48	0.30 (0)	0 (0.30)	0.75 (0)	0 (0.30)	0.30 (0)	0.95 (0)
72	0.55 (0)	0 (0.07)	0.2 (0)	0 (0.07)	0.55 (0)	4.85 (0)

Citral (geranial (**70**) and neral (**71**)) concentration also decreased over time in the absence of yeast, however both geranial (**70**) and neral (**71**) were detected at 48hr and 78hr (Table 4.3).

As citral concentration decreased with time in the absence of yeast, it suggests that the volatility of citral contributed to its decrease in concentration with increasing time. As citral was not detected after 48hr in the presence of yeast, it indicates that the yeast metabolised citral. The compounds citronellol (**69**), geraniol (**72**), nerol (**75**) and phenylethyl alcohol were detected in the presence of yeast, but not in the absence of yeast (Table 4.3), indicating these compounds are the products of metabolism. These results are consistent with research conducted by Chatterjee *et al*. (1999), who determined that citral is converted to geraniol (**72**) by yeast (*Saccharomyces cerevisae*). *Saccharomyces cerevisae* has also been used for the reduction of monoterpene aldehydes, such as the biotransformation of citronellal (**68**) to citronellol

(**69**), and for the reduction of other carbonyl compounds (Nielsen and Madsen, 1994; Poppe *et al*., 1991; Young and Ward, 1991).

Citronellol (**69**), nerol (**75**) and phenylethyl alcohol concentrations increased with time, which may be explained by the metabolism of citral with time. Geraniol (**72**) concentration increased up to 24hr, then decreased after 48hr (Table 4.3). This may be explained by the loss of geraniol (**72**) per well, due to its volatility or due to conversion to other metabolic products, such as citronellol (**69**). Citronellol (**69**) was detected as a metabolic product of geraniol (**72**) (Table 4.4). The conversion of citral to citronellol (**69**), geraniol (**72**) and nerol (**75**) may have contributed to the oestrogenic effects of citral observed in the yeast assay, as these compounds also demonstrated oestrogenic activity (Figures 4.8. and 4.9).

The potential oestrogenic activity of phenylethyl alcohol in the yeast assay requires further investigation. However, as the only metabolic product detected in the yeast culture treated with eugenol (**84**) was phenylethyl alcohol (Table 4.8) and eugenol (**84**) (including its metabolic products) did not demonstrate oestrogenic activity in the yeast assay (Figure 4.14), it is unlikely that phenylethyl alcohol contributed to the oestrogenic effects observed with citral, geraniol (**72**) or nerol (**75**) (see below).

Table 4.4. Assessment of metabolism of geraniol by yeast. Compounds detected in the presence and absence (*x*) of yeast. Peak area determined by ITD.

	Peak Area for Compound Detected		
Time (h)	**Citronellol**	**Geraniol**	**Phenylethyl Alcohol**
0	0 (0)	27.20 (24.65)	0 (0)
1	0 (0)	21.30 (23.60)	0 (0)
3	0 (0)	15.40 (17.30)	0 (0)
24	0.20 (0)	10.60 (12.85)	0.20 (0)
48	0.35 (0)	6.90 (7.15)	0.35 (0)
72	0.60 (0)	1.90 (1.95)	0.60 (0)

Table 4.5. Assessment of metabolism of nerol by yeast. Compounds detected in the presence and absence (*x*) of yeast. Peak area determined by ITD.

	Peak Area for Compound Detected		
Time (h)	**Citronellol**	**Nerol**	**Phenylethyl Alcohol**
0	0 (0)	28.15 (52.70)	0 (0)
1	0 (0)	22.20 (46.10)	0 (0)
3	0 (0)	19.80 (37.25)	0 (0)
24	0 (0)	14.00 (16.45)	0.15 (0)
48	0 (0)	6.90 (7.95)	0.30 (0)
72	0.10 (0)	1.10 (4.05)	1.10 (0)

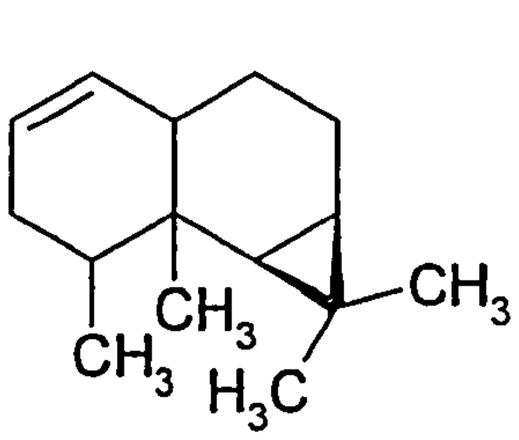
Geraniol (**72**) and nerol (**75**) concentrations also decreased over time in the presence and absence of yeast and geraniol (**72**) and nerol (**75**) concentrations were lower in the absence of yeast (Tables 4.4 and 4.5).

These results indicate that geraniol (**72**) and nerol (**75**) concentration decreased per well due to their volatility in the absence and presence of yeast, but concentrations were lower in the presence of yeast due to metabolism. Citronellol (**69**) and phenylethyl alcohol were detected in the wells containing the yeast culture, but not in the wells absent from yeast, and their concentrations increased over time (Tables 4.4 and 4.5). This indicates that geraniol (**72**) and nerol (**75**) were metabolised by yeast to give citronellol (**69**) and phenyethyl alcohol; citronellol (**69**) concentration was lower after treatment of the yeast with nerol (**75**), than with geraniol (**72**). Citronellol (**69**), which also demonstrated oestrogenic activity in the yeast assay (Figure 4.8), may have influenced the oestrogenic activity of geraniol (**72**) and nerol (**75**) in the yeast assay.

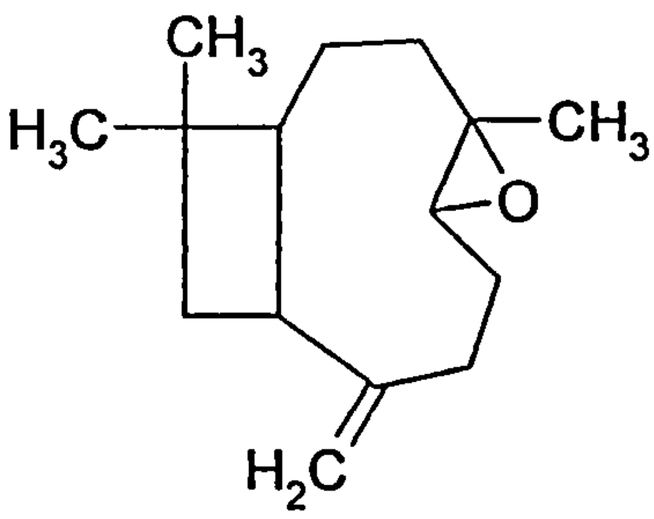
4.7.3 Assessment of Oestrogenic Activity of Some Sesquiterpenes Identified in *Melissa officinalis* Essential Oil, Using a Recombinant Yeast Screen

Of the sesquiterpenes assessed for oestrogenic activity using the recombinant yeast screen, nerolidol (**83**) ($p<0.001$) and *trans*-caryophyllene (**82**) ($p<0.05$) demonstrated significant oestrogenic activity (Figure 4.13). Treatment with caryophyllene oxide (**78**) caused a decrease in the absorbance reading with increasing concentration, indicating significant cytotoxic effects ($p<0.05$) (Figure 4.13).

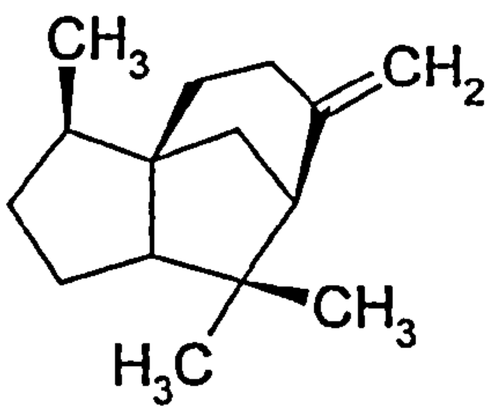
The inactivity of some of the hydrocarbon sesquiterpenes, such as (+)-calarene (**77**), (+)-β-cedrene (**79**), (-)-cubebene (**80**) and α-humulene (**81**) may be explained by their structural features, perhaps the lack of substituents required to act as a hydrogen bond donor in the LBD of hER. *Trans*-caryophyllene (**82**) does not contain any such substituent, but did demonstrate apparent oestrogenic effects in the yeast screen. *Trans*-caryophyllene (**82**) may have interacted with allosteric sites of the hER, other than the LBD. The sesquiterpene nerolidol (**83**), which demonstrated significant oestrogenic activity in the yeast screen (Figure 4.13), contains a hydroxyl substituent, which may have interacted favourably with the LBD of the hER to initiate an oestrogenic response. Again, it cannot be disregarded that the apparently inactive sesquiterpenes may have hER antagonistic properties.



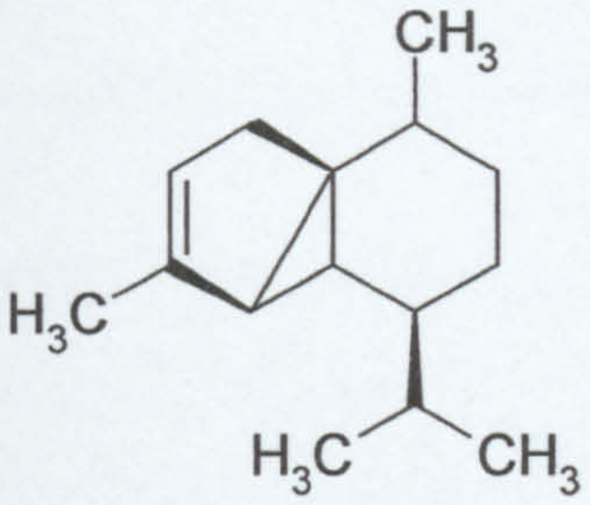
(+)-Calarene (**77**)



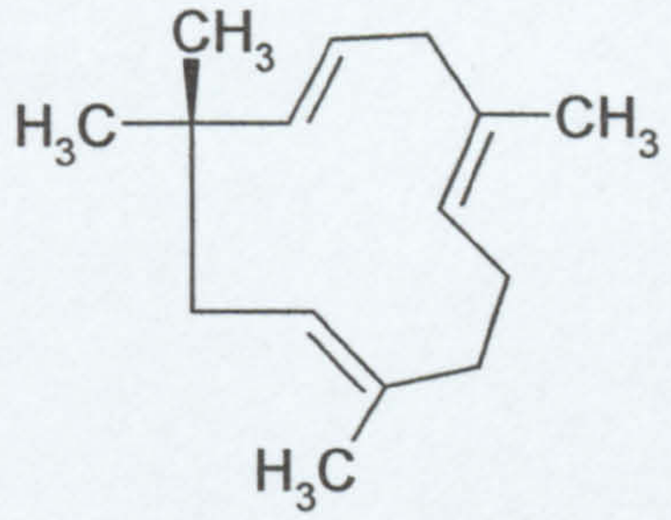
Caryophyllene oxide (**78**)



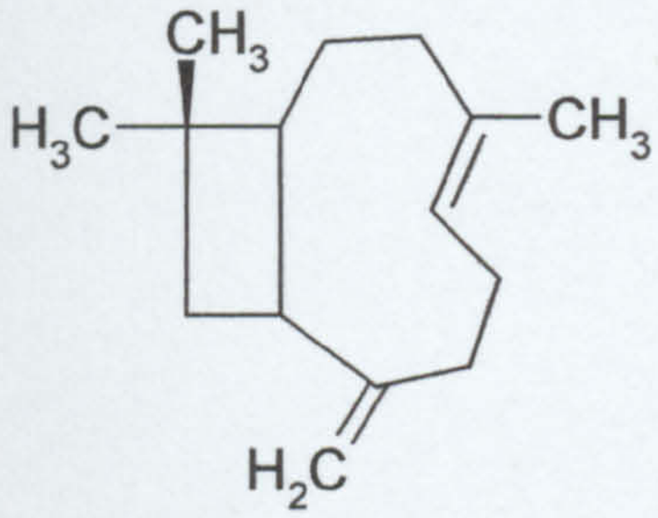
β-Cedrene (**79**)



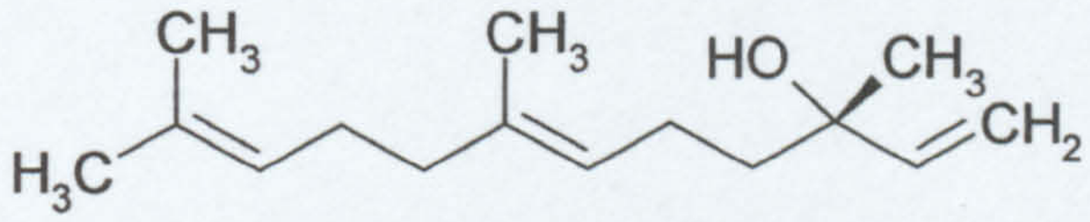
(-)-Cubebene (**80**)



α-Humulene (**81**)



Trans-Caryophyllene (**82**)



Nerolidol (**83**)

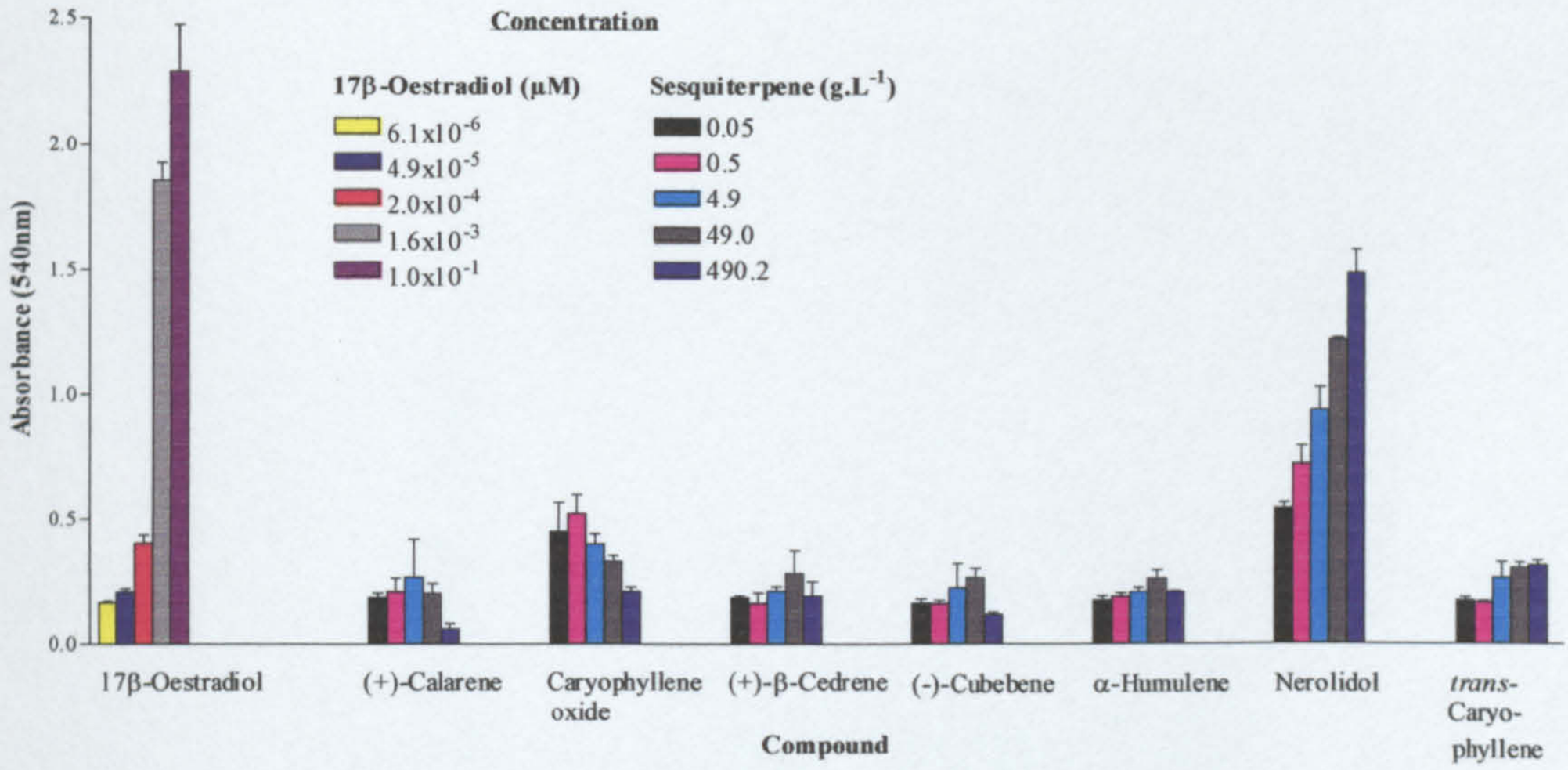
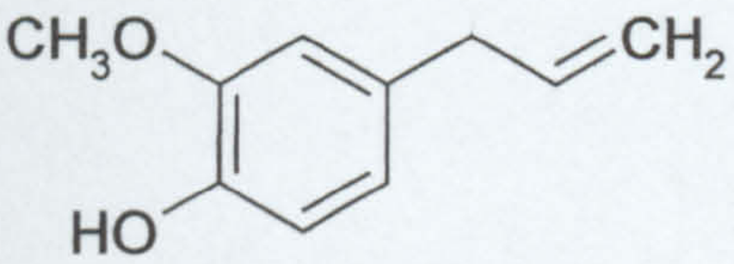


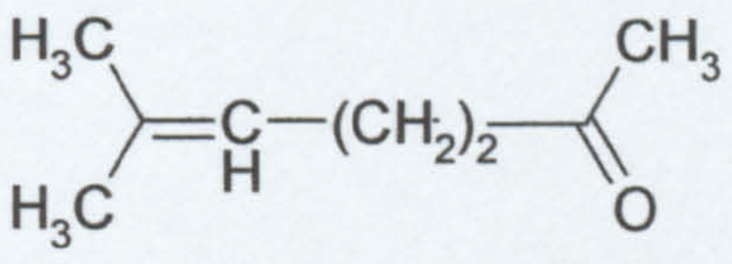
Figure 4.13. Oestrogenic activity of sesquiterpenes, (+)-calarene ($p>0.05$), caryophyllene oxide ($p<0.05$), (+)-β-cedrene ($p>0.05$), (-)-cubebene ($p>0.05$), α-humulene ($p>0.05$), nerolidol ($p<0.001$) and *trans*-caryophyllene ($p<0.05$), and 17β-oestradiol ($p<0.001$), assessed by stimulation of β-galactosidase activity in genetically modified yeast cells (n=3-6 ± SD).

4.7.4 Assessment of Oestrogenic Activity of Some Oil Constituents Identified in *Melissa officinalis* Essential Oil, Using a Recombinant Yeast Screen

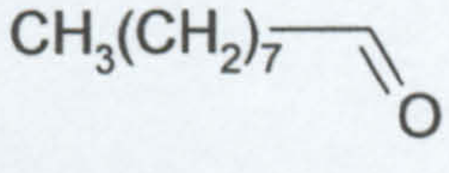
The aldehyde nonanal (**86**) ($p<0.01$) demonstrated significant oestrogenic activity in the yeast screen (Figure 4.14). This observation may be due to interaction of the aldehyde substituent with the LBD of the hER, although nonanal (**86**) may have influenced allosteric sites of the hER. The ketone 6-methyl-5-hepten-2-one (**85**) ($p>0.05$) and the phenylpropanoid eugenol (**84**) ($p>0.05$) did not demonstrate significant oestrogenic activity in the yeast screen (Figure 4.14). Eugenol (**84**) has recently been investigated for oestrogenic activity using the yeast bioassay, and was again found to be inactive (Miller *et al*., 2001).



Eugenol (**84**)



6-Methyl-5-hepten-2-one (**85**)



Nonanal (**86**)

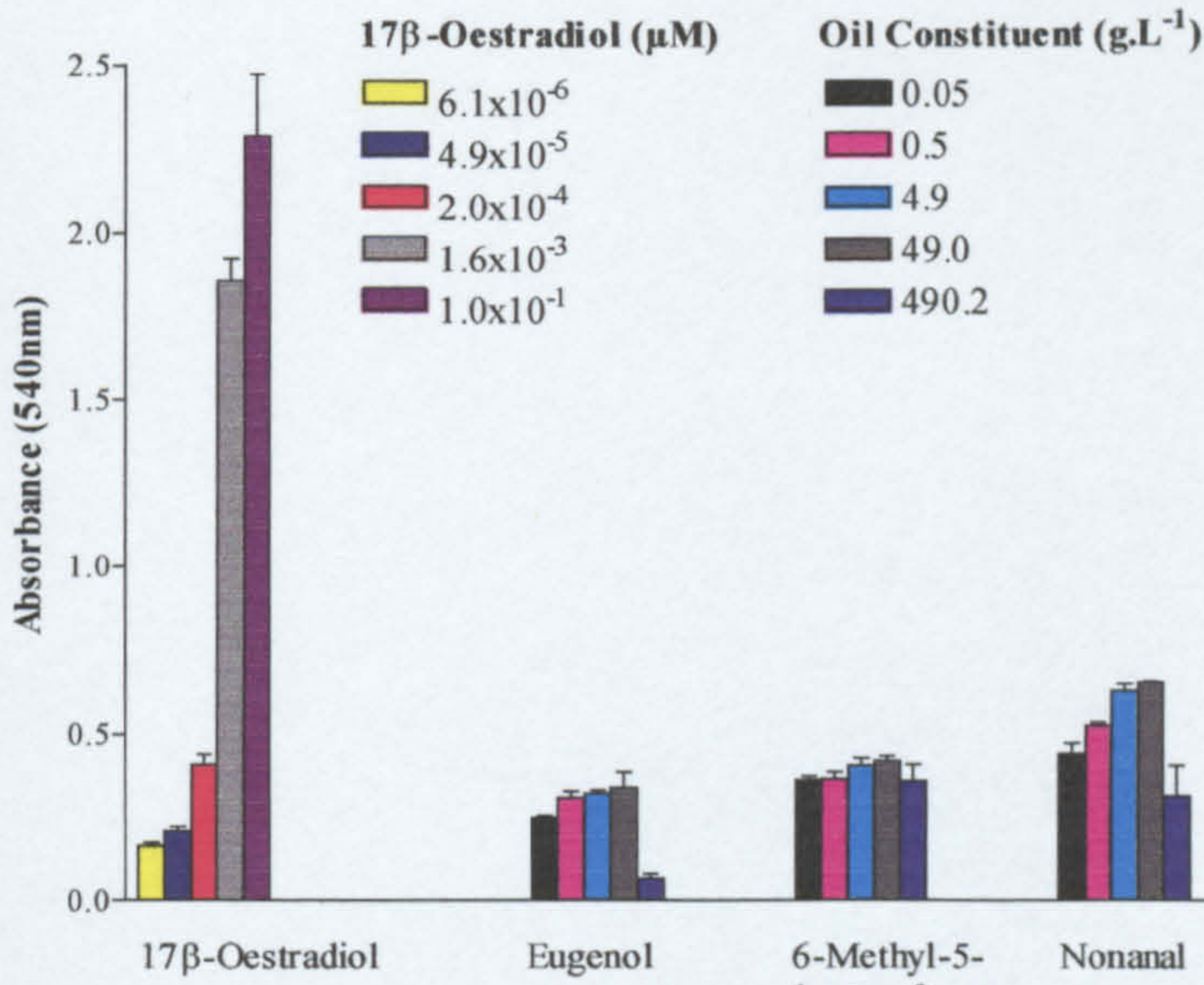


Figure 4.14. Oestrogenic activity of the *M. officinalis* oil constituents, eugenol ($p>0.05$), 6-methyl-5-hepten-2-one ($p>0.05$) and nonanal ($p<0.01$), and 17β-oestradiol ($p<0.001$), assessed by stimulation of β-galactosidase activity in genetically modified yeast cells (n=3-6 ± SD).

4.7.5 Assessment of Oestrogenic Activity of Citral and Geraniol, Using the Ishikawa Cell Line

The monoterpenes citral and geraniol (**72**) were two of the most active compounds in the yeast screen (refer to 4.7.1). Consequently, they were selected for further investigation of their oestrogenic potential in the mammalian Ishikawa cell line. Over the concentration range 0.2μM - 259.3μM, neither citral ($p<0.05$) nor geraniol (**72**) ($p<0.05$) showed significant oestrogenic activity in the Ishikawa cell line (Figure 4.15). Both compounds were cytotoxic at concentration ≥ 256.0μM (Figure 4.15). These results indicate that citral and geraniol (**72**) are not oestrogenic in the mammalian Ishikawa cells; inactivity in the Ishikawa cell line also occurred with the *M. officinalis* essential oil (refer to 4.6.3). It may also be possible that the monoterpenes citral and geraniol (**72**) were antagonistic in the Ishikawa cell line, so did not initiate an oestrogenic response.

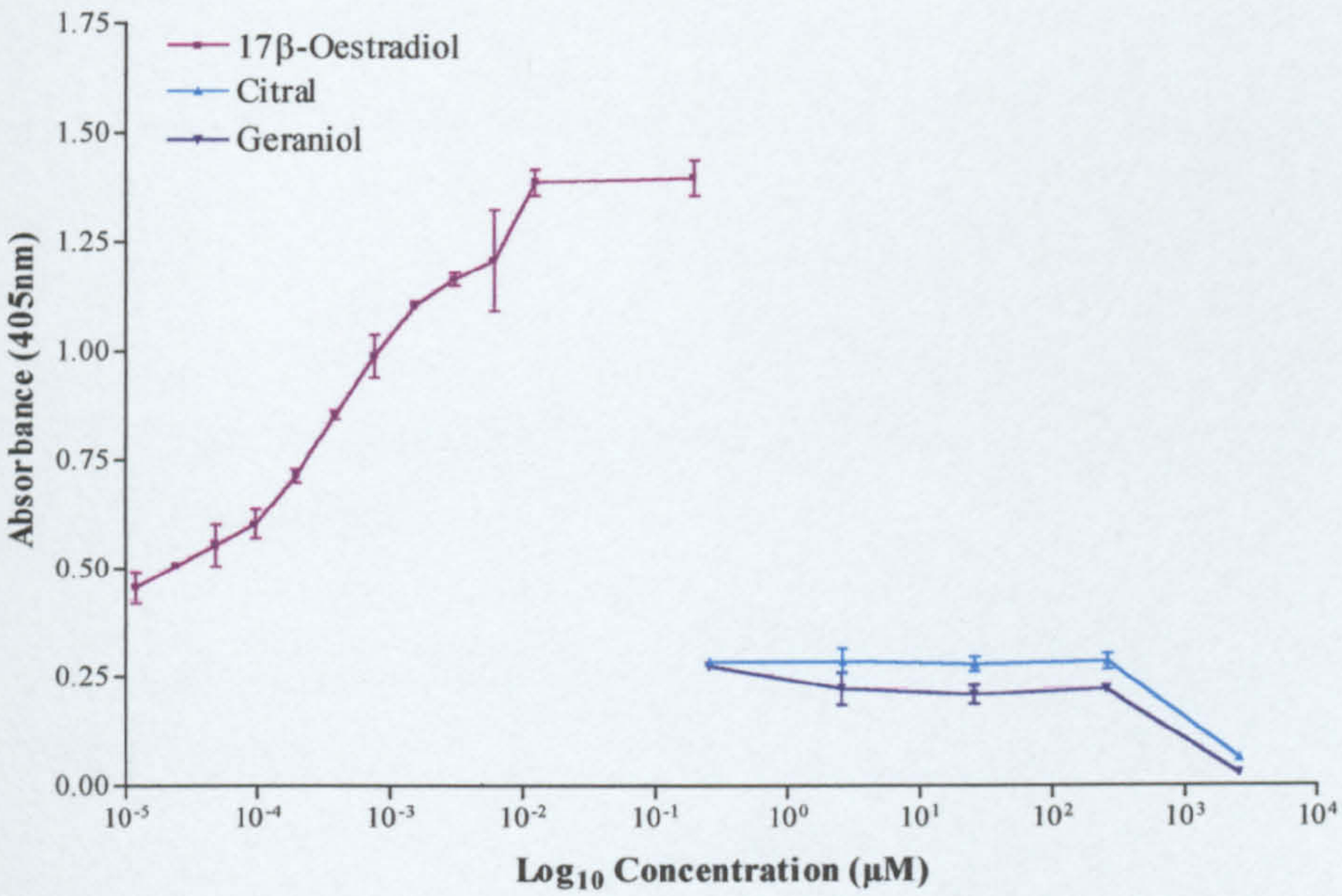


Figure 4.15. Oestrogenic activity of citral ($p<0.05$) and geraniol ($p<0.05$), and 17β-oestradiol ($p<0.001$), assessed by stimulation of alkaline phosphatase activity in Ishikawa cells (n=3-6 ± SD).

The mammalian cells may have different transcriptional mechanisms, or different metabolic activity to the yeast cells, so may have metabolised compounds rendering

them inactive. It is also possible that an oestrogenic response occurred at the cytotoxic concentration, so was undetected. The higher incubation temperature (37°C, as opposed to 32°C in the yeast screen) may have increased loss of volatile compounds, reducing the assay concentration required to initiate an oestrogenic response. Further investigations were considered necessary to establish any oestrogenic potential of these compounds, therefore experiments were extended to determine their affinity for the hER in receptor binding studies and their potential oestrogenic activity *in vivo* (refer to 4.8 and 4.9).

Inconsistent results between different *in vitro* assays have been identified previously, and may be attributed to different metabolic capabilities of the assay systems (Petit *et al.*, 1997; Shelby *et al.*, 1996). ERβ expression is reported to be relatively high in Ishikawa cells but ERα is reported to be predominant in the recombinant yeast cells (Dechering *et al.*, 2000). The monoterpenes citral and geraniol (**72**), and indeed the other essential oil constituents present in *M. officinalis* essential oil, may have greater affinity for ERα than ERβ, which may explain the apparent oestrogenic response observed in the yeast screen, which was not apparent in the Ishikawa cells. Receptor binding studies for ERα and ERβ were conducted to investigate this possibility (refer to 4.8).

4.8 Results and Discussion: Oestrogen Receptor Binding Investigations of Essential Oil Constituents

4.8.1 Assessment of Oestrogen Receptor Binding of Essential Oil Constituents, Using Ishikawa Cells

4.8.1.1 Assessment of Oestrogen Receptor Binding of Geraniol, Using Ishikawa Cells

Geraniol (**72**) ($p<0.01$) significantly displaced [^{3}H]-17β-oestradiol from Ishikawa cell ERs over the concentration range 0.06mM - 6.0mM (Figure 4.16).

Agonistic and antagonistic activities cannot be distinguished, however this result is further evidence to suggest that geraniol (**72**) has an affinity for the ER. This result could also be explained by geraniol (**72**) altering the conformation of the ER but not binding to the LBD, which may also inhibit [^{3}H]-17β-oestradiol binding. Therefore

the effect of other essential oil constituents, which did not demonstrate oestrogenic activity in the yeast screen, was investigated for their affinity for Ishikawa cell ERs (refer to 4.8.1.2).

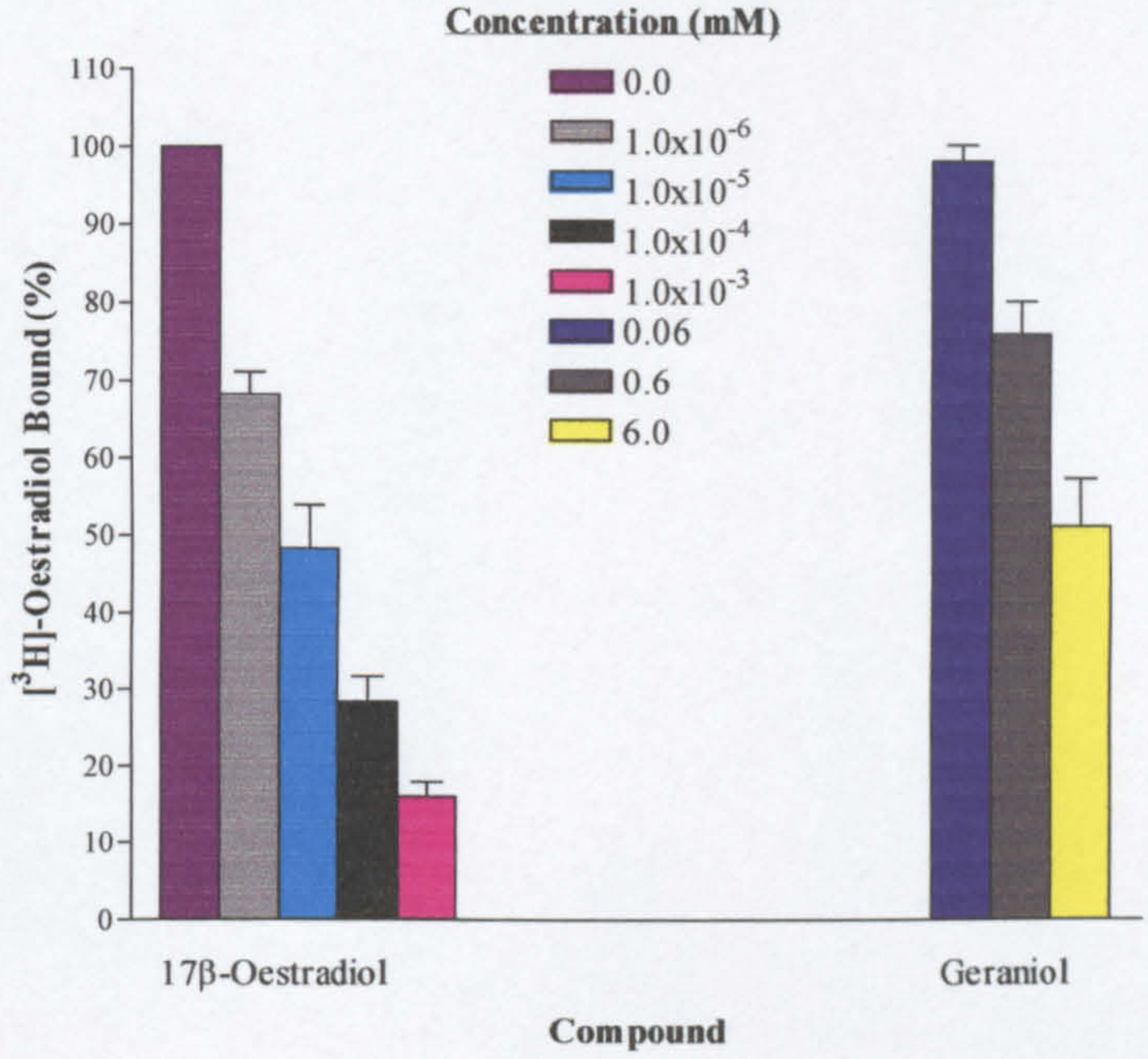


Figure 4.16. Competitive displacement of [^{3}H]-17β-oestradiol from Ishikawa cell oestrogen receptors by geraniol (p<0.01) and 17β-oestradiol (p<0.001) (n=3-6 ± SD).

4.8.1.2 Assessment of Oestrogen Receptor Binding of 1, 8-Cineole, Eugenol, Geraniol and 6-Methyl-5-hepten-2-one, Using Ishikawa Cells

The essential oil constituents, which did not demonstrate oestrogenic activity in the yeast screen (refer to 4.7), were selected because of their similar chemical properties to geraniol (**72**) (d: 0.889; mw: 154.25) to if investigate the effect of essential oil constituents on displacement of [^{3}H]-17β-oestradiol binding to ERs was selective. The compounds selected were 1, 8-cineole (**56**) (d: 0.921; rmm: 154.25), eugenol (**84**) (d: 1.066; rmm: 164.25) and 6-methyl-5-hepten-2-one (**85**) (d: 0.855; rmm: 126.2).

By comparing the essential oil constituents for their displacement of [^{3}H]-17β-oestradiol from ERs, it appears that geraniol (**72**) may be selective for [^{3}H]-17β-oestradiol displacement. This may be due to interaction with the LBD of the ER. The results show that 1, 8-cineole (**56**) (p>0.05) and 6-methyl-5-hepten-2-one (**85**) (p>0.05) did not significantly displace [^{3}H]-17β-oestradiol from ERs and only

displaced [^{3}H]-17β-oestradiol binding by 5.2% and 1.0% respectively at 6.0mM (Figure 4.17). These results are consistent with the results from the yeast screen, which indicated that 1, 8-cineole (**56**) and 6-methyl-5-hepten-2-one (**85**) did not initiate a significant oestrogenic response via interaction with the ER (refer to 4.7).

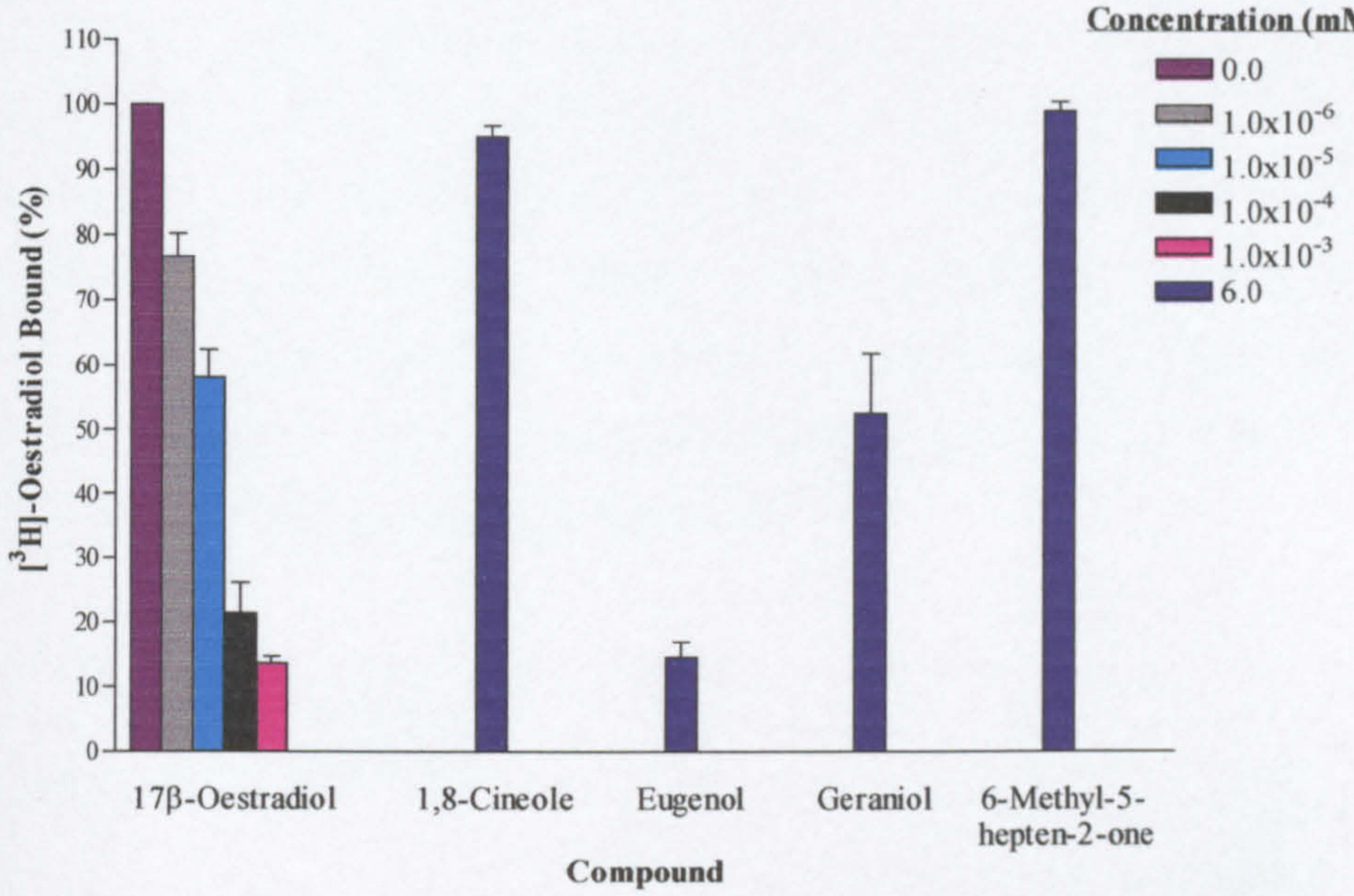


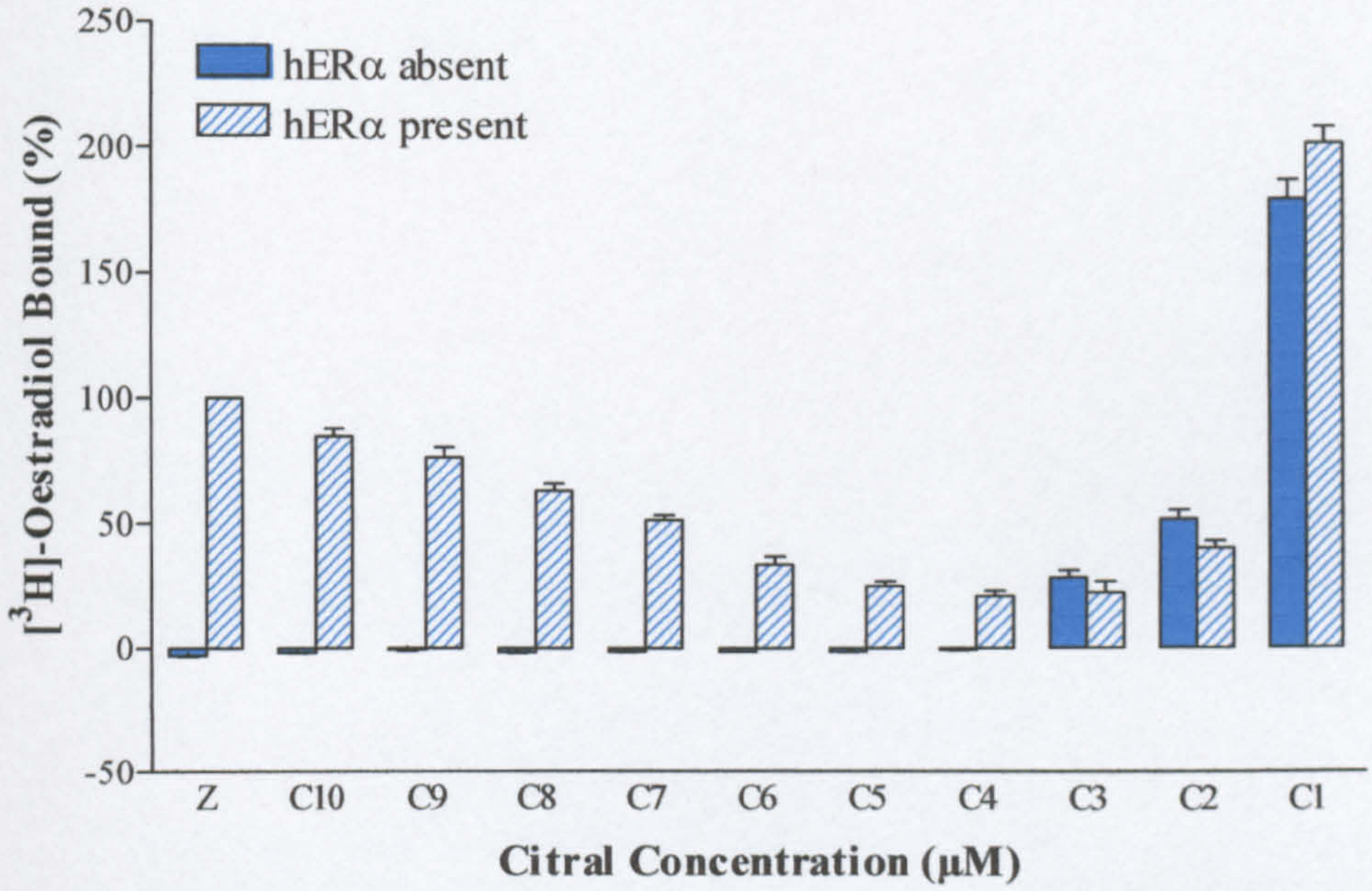
Figure 4.17. Competitive displacement of [^{3}H]-17β-oestradiol from Ishikawa cell oestrogen receptors by the essential oil constituents 1, 8-cineole ($p>0.05$), eugenol ($p<0.01$), geraniol ($p<0.01$) and 6-methyl-5-hepten-2-one ($p>0.05$), and 17β-oestradiol ($p<0.001$) (n=3-6 ± SD).

Geraniol (**72**) ($p<0.01$) significantly displaced [^{3}H]-17β-oestradiol binding to ERs by 47.6% at 6.0mM (Figure 4.17). This result is also consistent with the results from the yeast screen, which indicated geraniol (**72**) may bind to the ER to initiate an oestrogenic reponse (refer to 4.7).

Eugenol (**84**) ($p<0.01$) significantly displaced [^{3}H]-17β-oestradiol binding to ERs by 85.5% at 6.0mM (Figure 4.17), but did not demonstrate significant oestrogenic activity in the yeast screen (refer to 4.7.4). This could be explained by eugenol (**84**) acting as an anti-oestrogen. The potential ER antagonist properties of eugenol (**84**) were further investigated by evaluating its effect on the oestrogenic response initiated by E2 (**14**), using the yeast screen (refer to 4.10).

4.8.2 Assessment of α- and β-Oestrogen Receptor Binding of Citral, Eugenol, Geraniol, and Nerol

Citral, eugenol (**84**), geraniol (**72**) and nerol (**75**) were analysed for their ability to displace [^{3}H]-17β-oestradiol binding to isolated ERα and ERβ over the concentration range 201.0μM - 115930.1μM. However, at the three highest concentrations for each test compound (25729.3μM - 115930.1μM) exceptionally high CPM values were generated but, at the concentrations 201.0μM - 14491.3μM, dose dependent displacement of [^{3}H]-17β-oestradiol binding to ERα and ERβ was observed (Figures 4.18 - 4.21). To investigate this occurrence, the assays were repeated, but were extended to evaluate the effect of the test compound on [^{3}H]-17β-oestradiol in the absence of ERα and ERβ as an appropriate control.

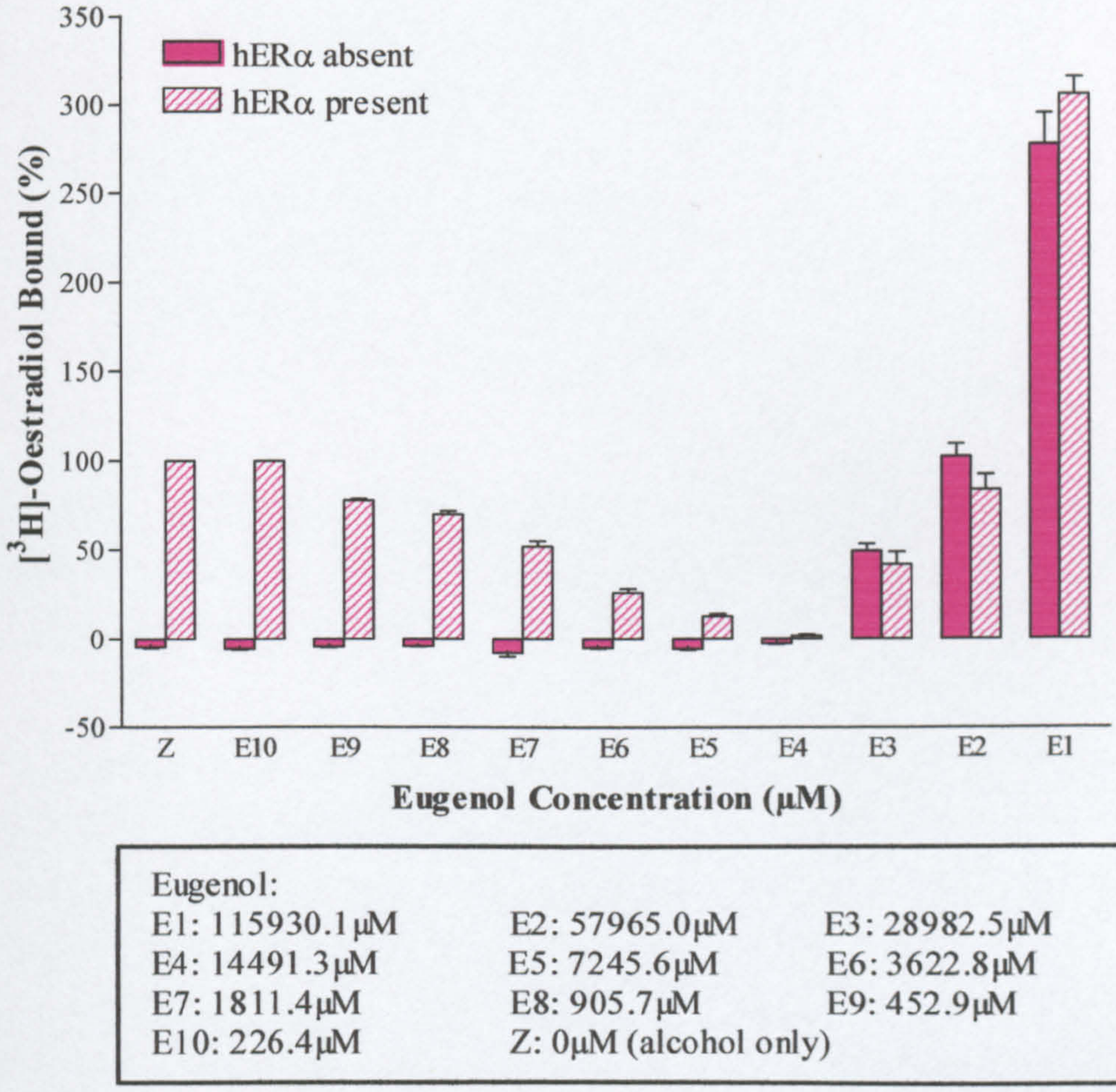


Citral:

C1: 104158.9μM	C2: 52079.4μM	C3: 26039.7μM
C4: 13019.9μM	C5: 6509.9μM	C6:3255.0μM
C7: 1627.5μM	C8: 813.7μM	C9: 406.9μM
C10: 203.4μM	Z: 0μM (alcohol only)	

Figure 4.18. Competitive displacement of [^{3}H]-17β-oestradiol from α-oestrogen receptors by citral (p<0.001) and 17β-oestradiol (p<0.001) and the effect of citral in the assay, in the absence of α-oestrogen receptors (n=3-6 ± SD).

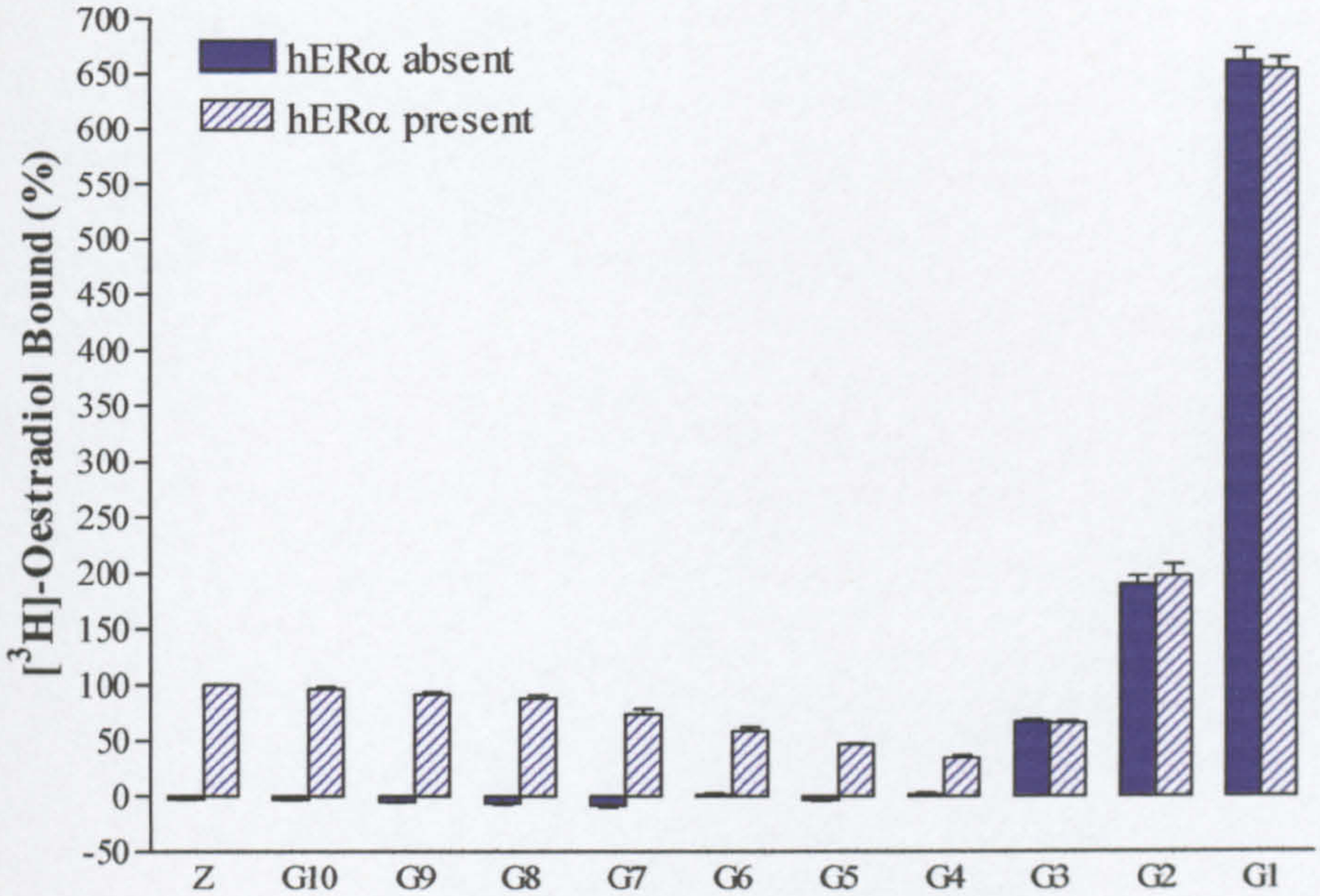
The results show that for all four test compounds, the exceptionally high CPM values were generated both in the presence and absence of ERα (Figures 4.18 - 4.21). At concentrations 201.0μM - 14491.3μM dose dependent displacement of [^{3}H]-17β-oestradiol binding was observed in the presence of ERα, but in the absence of any receptor, high CPM values were not observed (Figures 4.18 - 4.21). This indicates that the test compounds did not interfere in the assays at low concentrations, but at higher concentrations (>25729.3μM) did interfere in the assays. This could be due to the high concentrations of test compounds being at sufficient levels to precipitate [^{3}H]-17β-oestradiol.



Eugenol:

E1: 115930.1μM	E2: 57965.0μM	E3: 28982.5μM
E4: 14491.3μM	E5: 7245.6μM	E6: 3622.8μM
E7: 1811.4μM	E8: 905.7μM	E9: 452.9μM
E10: 226.4μM	Z: 0μM (alcohol only)	

Figure 4.19. Competitive displacement of [^{3}H]-17β-oestradiol from α-oestrogen receptors by eugenol (p<0.001) and 17β-oestradiol (p<0.001) and the effect of eugenol in the assay, in the absence of α-oestrogen receptors (n=3-6 ± SD).



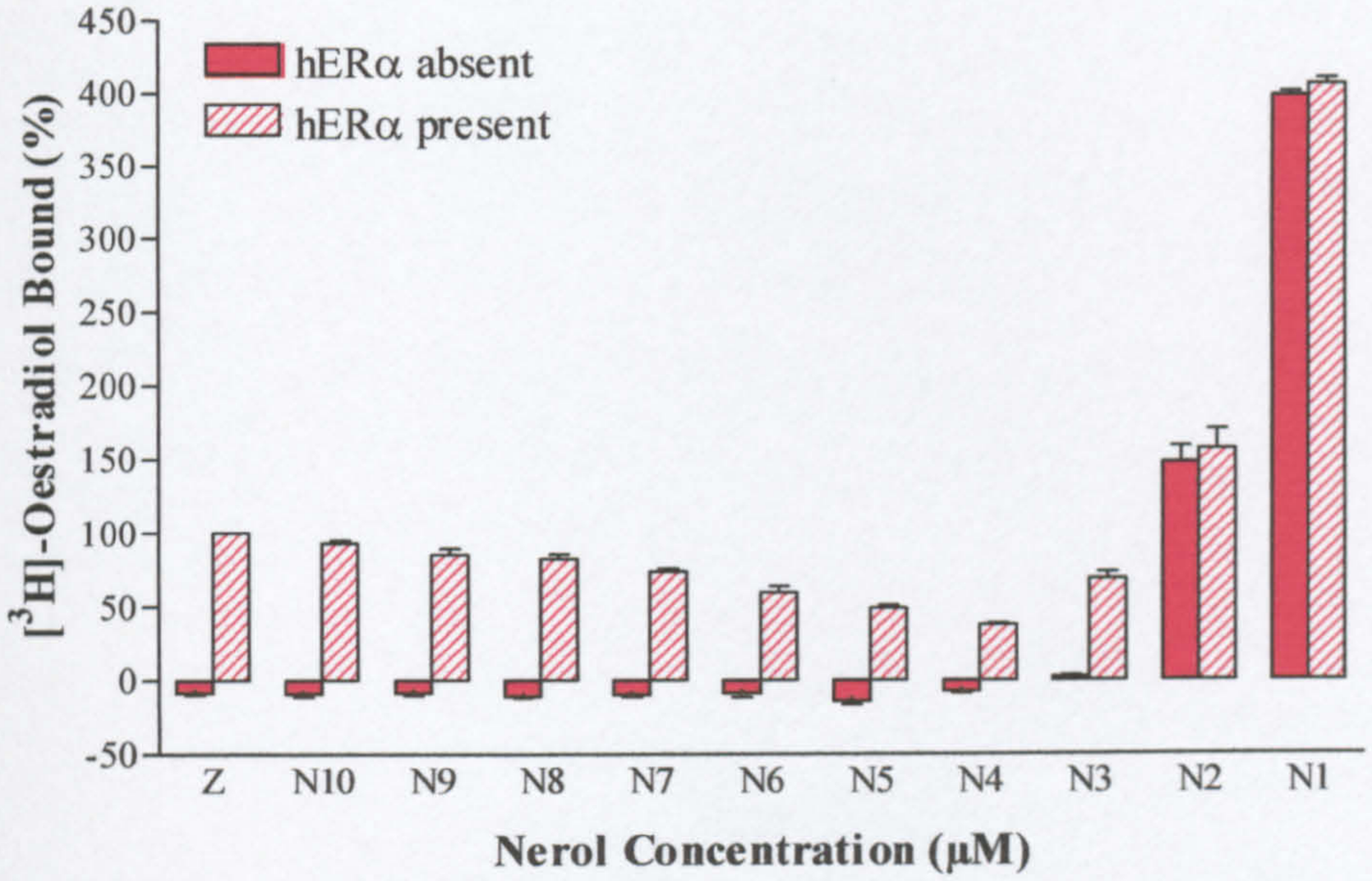
Geraniol:

G1: 102917.3 μM	G2: 51458.7 μM	G3: 25729.3 μM
G4: 12864.7 μM	G5: 6432.3 μM	G6:3216.2 μM
G7: 1608.1 μM	G8: 804.0 μM	G9: 402.0 μM
G10: 201.0 μM	Z: 0 μM (alcohol only)	

Figure 4.20. Competitive displacement of [^{3}H]-17β-oestradiol from α-oestrogen receptors by geraniol (p<0.001) and 17β-oestradiol (p<0.001) and the effect of geraniol in the assay, in the absence of α-oestrogen receptors (n=3-6 ± SD).

The concentrations of citral, eugenol (**84**), geraniol (**72**) and nerol (**75**) required to displace [^{3}H]-17β-oestradiol binding were 10^4 - 10^6-fold greater than the E2 (**14**) concentrations required. This indicates that the test compounds have only weak affinity for ERα and ERβ, relative to E2 (**14**).

Citral, eugenol (**84**), geraniol (**72**) and nerol (**75**) showed greater affinity for ERα than ERβ (Figures 4.22 and 4.23; Table 4.6). This may be explained by the structures and LBDs of ERα and ERβ being different (Dechering *et al.*, 2000; Kuiper *et al.*, 1997; Mosselman *et al.*, 1996; Paech *et al.*, 1997). As the test compounds showed different affinities for ERα and ERβ, they may act specifically to displace [^{3}H]-17β-oestradiol binding, perhaps by having greater affinity for the LBD of ERα than ERβ.



Nerol:

N1: 102917.3μM	N2: 51458.7μM	N3: 25729.3μM
N4: 12864.7μM	N5: 6432.3μM	N6:3216.2μM
N7: 1608.1μM	N8: 804.0μM	N9: 402.0μM
N10: 201.0μM	Z: 0μM (alcohol only)	

Figure 4.21. Competitive displacement of [^{3}H]-17β-oestradiol from α-oestrogen receptors by nerol (p<0.001) and 17β-oestradiol (p<0.001) and the effect of nerol in the assay, in the absence of α-oestrogen receptors (n=3-6 ± SD).

Table 4.6. Affinity (EC_{50} values) of citral, eugenol, geraniol, nerol and 17β-oestradiol (E2) for isolated ERα and ERβ.

Compound	**ERα: EC_{50}**	**ERβ: EC_{50}**
Citral	1754.4μM	5610.5μM
Eugenol	1966.2μM	5603.5μM
Geraniol	5576.6μM	>12864.7μM
Nerol	6127.8μM	>12864.7μM
E2	21.1nM	16.9nM

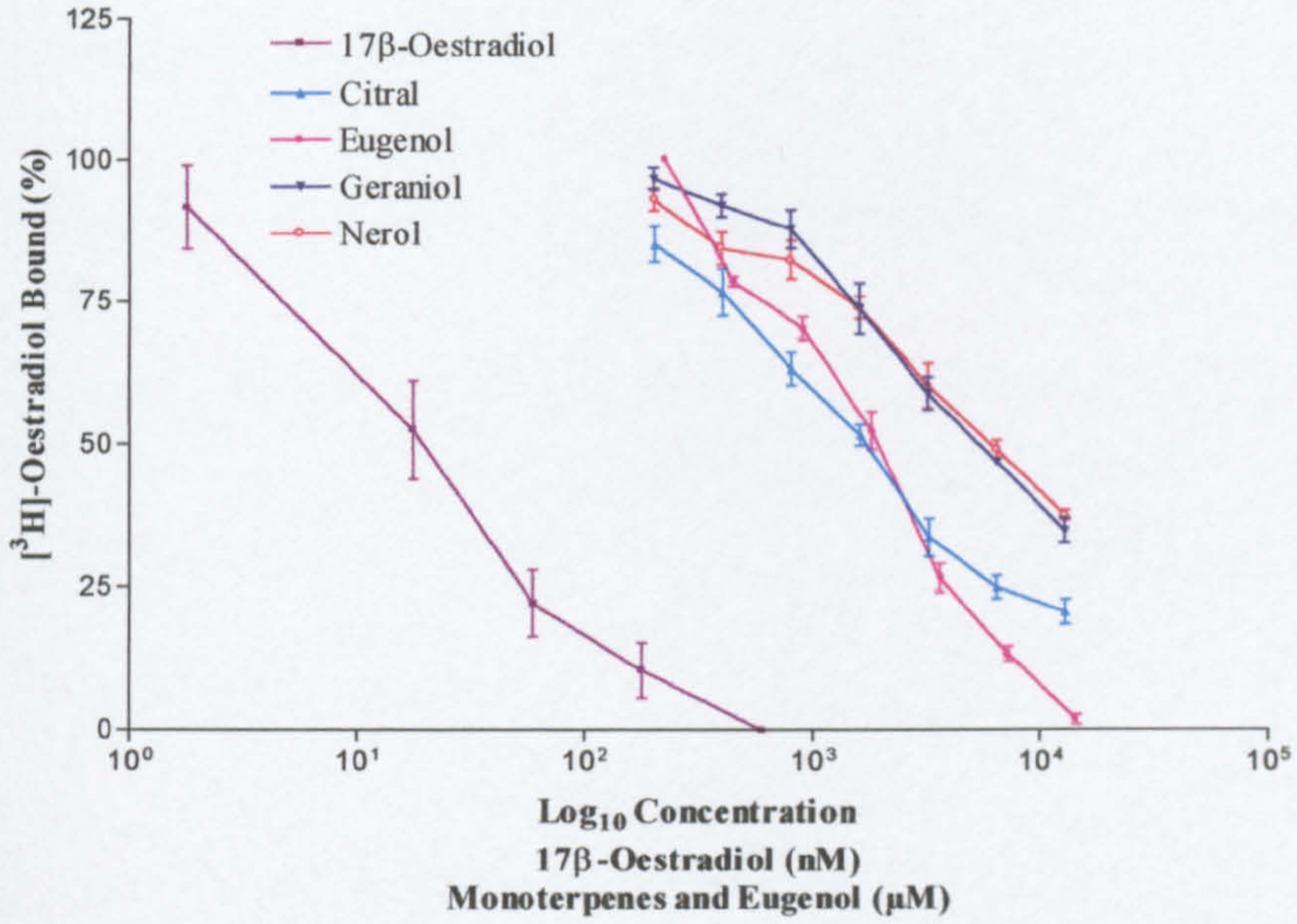


Figure 4.22. Competitive displacement of [^{3}H]-17β-oestradiol from α-oestrogen receptors by citral (p<0.001), eugenol (p<0.001), geraniol (p<0.001) and nerol (p<0.001), and 17β-oestradiol (p<0.001) (n=3-6 ± SD).

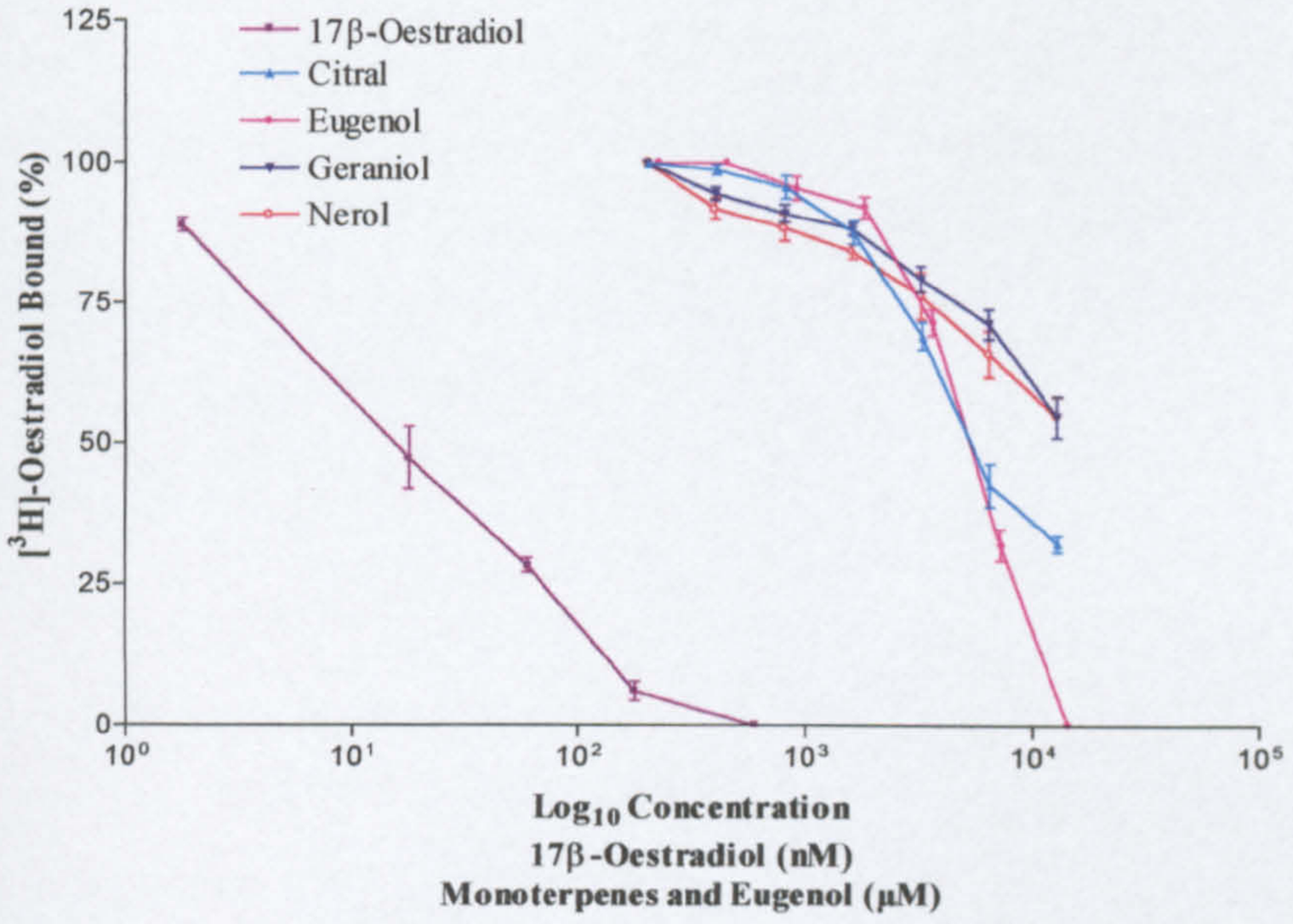


Figure 4.23. Competitive displacement of [^{3}H]-17β-oestradiol from β-oestrogen receptors by citral (p<0.001), eugenol (p<0.001), geraniol (p<0.001) and nerol (p<0.001), and 17β-oestradiol (p<0.001) (n=3-6 ± SD).

The test compounds showing greater selectivity for ERα than ERβ, may explain why the monoterpenes citral and geraniol (**72**) showed oestrogenic activity in the yeast assay which contains predominantly ERα (refer to 4.7.1), but did not show oestrogenic activity in the Ishikawa cell assay, which contains predominantly ERβ (refer to 4.7.5).

Eugenol (**84**) displaced [^{3}H]-17β-oestradiol binding to both ERα than ERβ, again suggesting that eugenol (**84**) may have the potential to be a competitive antagonist.

The monoterpenes showed greater affinity for ERα than ERβ (Figures 4.22 and 4.23; Table 4.6), unlike other phyto-oestrogens (e.g. genistein (**89**)), which are reported to have greater affinity for ERβ (Mason, 2001). ERβ is reported to predominate in the prostate, bone and vascular tissue but, ERα is reported to predominate in the breast, uterus and ovary (Dechering *et al.*, 2000; Kuiper *et al.*, 1997; Mason, 2001). This suggests that ERα selective compounds, perhaps the monoterpenes, may promote adverse effects in breast tissue, uterine tissue and ovaries, such as carcinogenesis.

ERβ is reported to mediate the beneficial effects of oestrogens on learning and memory (Fillit, 1994; Shughrue *et al.*, 1997; Wickelgren, 1997). However, both ERα and ERβ have been detected in the hippocampus, an area important for cognition (Osterlund *et al.*, 1998; Register *et al.*, 1998). More recently, ERα has also been detected in rat and human cerebral cortex (Butler *et al.*, 1999; Osterlund *et al.*, 2000). Therefore ERα may also be relevant in mediating the beneficial effects of oestrogens on cognition, but further investigations are necessary regarding the relationship between ERα and ERβ within the CNS and cognition. The potential adverse effects of ERα agonists also requires further assessment before any recommendations regarding their use for management of AD.

4.9 Results and Discussion: Assessment of Oestrogenic Activity of Citral and Geraniol *in vivo*

4.9.1 Assessment of Oestrogenic Activity of Citral and Geraniol, Using a Uterotrophic Assay

The uterotrophic assay requires the potential oestrogen to stimulate uterine growth (Martin *et al.*, 1976; Odum *et al.*, 1997; Shelby *et al.*, 1996). Administration of E2 (**14**) gave a 4.8-fold increase in uterine weight, compared to the control (alcohol only). However, after administration of the two compounds citral and geraniol (**72**), no evidence of an increase in uterine weight was observed compared to the control (Figure 4.24).

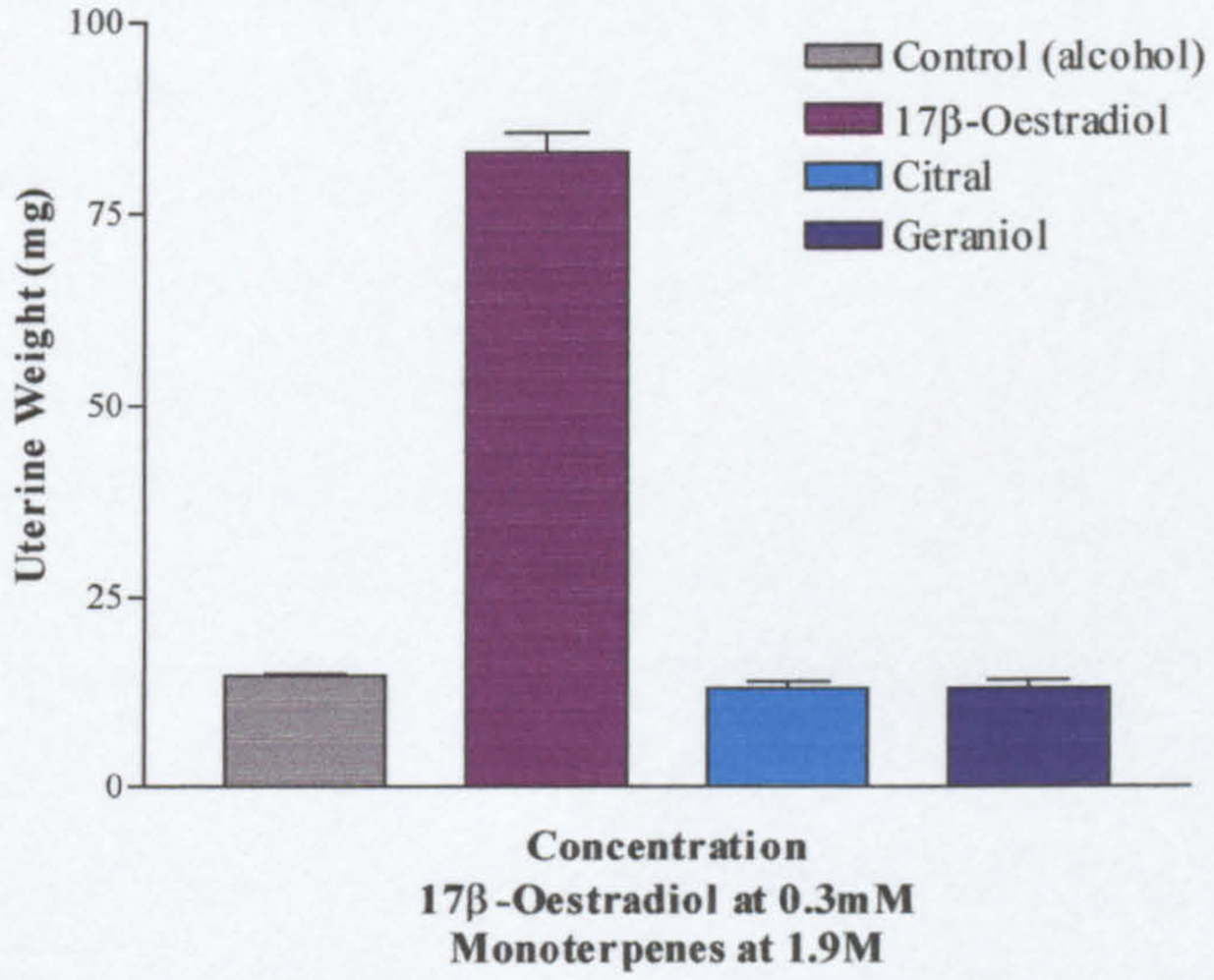


Figure 4.24. Effect of alcohol (control), citral, geraniol and 17β-oestradiol on uterine weight, following transdermal administration twice daily for 3 days to ovariectomised mice (uterotrophic assay) (n=5 ± SEM).

ERβ expression is reported to be low in the uterus (Dechering *et al.*, 2000), which indicates that ERα is responsible for mediating the uterotrophic response. The apparent lack of oestrogenic activity of these monoterpenes *in vivo* may be due to low affinity for ERα in uterine tissue, since although citral and geraniol (**72**) showed apparently weak oestrogenic activity in the yeast screen, in which ERα predominates

(refer to 4.7.1) and greater affinity for ERα than ERβ in the receptor binding studies (refer to 4.8.2), the binding affinity of citral and geraniol (**72**) for ERα was much less potent than E2 (**14**) affinity (refer to 4.8.2).

It has been reported that weak oestrogens stimulate uterine biochemical responses to a minimal degree (Clark and Peck, 1979). Therefore any oestrogenic effect of the monoterpenes may have been too small for detection, and may have required assessment over a longer time period. Bolus administration of the oestrogenic compound estriol did not initiate an increase in uterine weight in female ovariectomised rats (Clark *et al.*, 1977). This was attributed to the low retention time of the estriol-ER complexes but, continuous administration of estriol (via implants) did initiate a uterine growth response (Clark *et al.*, 1977). This result indicates that estriol may not be a weak oestrogen, but could be regarded as a short-term oestrogen. The negligible uterotrophic activity of estriol *in vivo* has also been suggested to have occurred due to rapid elimination from the animal (Korach *et al.*, 1980; Kupfer, 1987). Similarly, these occurrences may explain the lack of response with the monoterpenes in the uterotrophic assay. Perhaps more frequent administration of each compound may have permitted constant systemic concentrations, which may have induced an oestrogenic response.

Citral is rapidly metabolised and excreted, with no long term storage in the rat or mouse; following oral administration, ^{14}C labelled citral is rapidly absorbed and excreted within 72hr by the rat, and within 120hr by the mouse (Phillips *et al.*, 1976). The monoterpenes may also have been rapidly metabolised to other compounds that are not oestrogenic *in vivo*. Two of the major metabolites identified in urine after administration of citral to the rabbit were hildebrandt acid and dihydro-hildebrandt acid (Ishida *et al.*, 1989; Williams, 1959). Hildebrandt acid has also been identified as a metabolite of geraniol (**72**) in the rat suggesting that similar metabolic pathways occur to those in the rabbit (Chadha and Madyastha, 1984). It would be useful to evaluate the oestrogenic activity of these metabolites in other oestrogenic assays.

Citral has previously been reported to induce local hyperkeratosis following repeated application to the same area of skin (Abramovici *et al.*, 1987), which raises the possibility that this response may have interfered with transdermal absorption in the assays in the present study. The relative oestrogenic potency of the monoterpenes and E2 (**14**) may reflect the rate of transdermal absorbtion of each compound. Impairment

of transdermal absorption by hyperkeratosis may have reduced the systemic concentrations of the monoterpenes required to promote uterine growth.

4.9.2 Assessment of Oestrogenic Activity of Citral and Geraniol, Using an Acute Assay

A quantitative index of the permeability of the uterine vasculature was obtained from the leakage of radiolabelled albumin after the application of E2 (**14**) or the test substance. Following transdermal administration of E2 (**14**), changes in uterine vascular permeability enabled significant promotion of radiolabelled albumin leakage, compared to the vascular changes in the control tissue (muscle) (Table 4.7). However, no such vascular changes were observed with the control (alcohol only), or with citral or geraniol (**72**) (Table 4.7).

Table 4.7. Uterine weight and vascular permeability 4hr following transdermal application of 100µl alcohol, or 17β-oestradiol, citral or geraniol (diluted in alcohol). Data are presented as mean ± SEM. ** $p<0.01$ compared to the control (alcohol).

Treatment	No. of mice	Uterine Weight (mg)	Uterine Vascular Permeability (EAV)	Muscle Vascular Permeability (EAV)
Control (alcohol)	5	13.5 ± 0.28	5.96 ± 0.54	1.24 ± 0.04
17β-Oestradiol	5	19.0 ± 0.15**	20.3 ± 1.12**	0.94 ± 0.03
Citral	5	12.5 ± 0.88	12.5 ± 4.31	0.95 ± 0.04
Geraniol	5	12.8 ± 0.59	4.72 ± 0.21	1.17 ± 0.13

The failure of any uterotrophic effect may have reflected the need to have continuous exposure to a weak oestrogen to sustain a uterine growth response (Martin *et al.*, 1976), but this limitation did not apply to the more acute vascular permeability assay. The uterine vascular permeability assay is an acute, rapid and easily monitored response of the uterus to oestrogenic stimulation (Arvidson, 1977). Immediate vasoactive effects on the ventral prostate of adolescent rats following a single transdermal dose of citral (22.8mg/150g rat) to the lumbodorsal region of skin have

been reported to occur (Scolnik *et al.*, 1994). The mechanism of action for this effect was unknown, but may have been due to the initial stages of a neoplastic effect.

Due to the volatile nature of citral and geraniol (**72**), the amounts of the monoterpenes reaching the systemic circulation is likely to have been considerably less than was actually applied, which may have contributed to a sub-optimal dose of citral being present in the systemic circulation. This occurrence may explain the apparent lack of oestrogenic activity of the monoterpenes *in vivo*.

It has been reported that geraniol (**72**) was not absorbed through the intact shaved abdominal skin of the mouse after 2hr (Meyer and Meyer, 1959), which could also explain the lack of oestrogenic effects of geraniol (**72**) *in vivo* in the present study. However, dermal application of 20mg or more of citral daily (or less frequently) is an established model for inducing prostatic hyperplasia *in vivo*, but these studies involved citral administration for periods of 1 - 3 months (Abramovici *et al.*, 1987; Geldof *et al.*, 1992; Massas *et al.*, 1991; Servadio *et al.*, 1986). This suggests that prolonged and continuous administration of the monoterpenes may be required to promote an oestrogenic response *in vivo*. Such doses compare to 29.6mg citral/mouse (acute assay) and only 14.8mg citral/mouse (uterotrophic assay) per application in the present study.

The mechanisms by which citral induces prostatic hyperplasia in rats remains uncertain. Synergism between citral and testosterone has been proposed to explain hyperplastic changes in the rat ventral prostate (Engelstein *et al.*, 1996), and an inflammatory response was also suggested to explain the effects of citral on rat prostate (Scolnik *et al.*, 1994). It has also been suggested that the proliferative effects observed in the prostate following administration of citral may be due to an oestrogenic effect (Geldof *et al.*, 1992). Oestrogen receptors have been identified in human prostate (Schulze and Claus, 1990) and E2 (**14**) has been linked to the development of prostatic hyperplasia (Habenicht and El Etreby, 1988). In addition, the application of citral directly to the vagina of ovariectomised rats increased the proliferation of the vaginal epithelium, similar to the effect of E2 (**14**) (Geldof *et al.*, 1992). It was also reported that citral inhibited E2 (**14**) binding to ERs using rat uterine cytosol, while no such inhibition was observed with testosterone for androgen receptors (Geldof *et al.*, 1992). However, the evidence of oestrogenic activity from these studies by Geldof *et al.* (1992) was not conclusive. The mitotic responses in the vagina may have occurred due to local keratinisation effects of citral, which may

reflect hypertrophy that is not associated with oestrogenic activity, and the data on competition with E2 (**14**) for oestrogen receptors was very limited (tested at only three concentrations).

It is apparent that further investigations regarding the mode of action of the monoterpenes citral and geraniol (**72**) are necessary, to explain their reported effects on induction of prostatic hyperplasia and proliferation of vaginal epithelium, and to confirm absence of effects on uterine weight over an extended period of time.

4.10 Results and Discussion: Assessment of Anti-Oestrogenic Activity of Eugenol

4.10.1 Assessment of Anti-Oestrogenic Activity of Eugenol, Using the Recombinant Yeast Screen

Oestrogenic compounds that bind to the ER are reported to initiate ER dimerisation and binding to oestrogen response elements (ERE) on DNA, with subsequent interaction with transcription factors, which modulates gene transcription (Beekman *et al.*, 1993; Dechering *et al.*, 2000; Diel *et al.*, 1998; Parker, 1993; Ryffel *et al.*, 1988). Anti-oestrogens may compete with an oestrogenic compound and bind to the ER, but do not activate gene transcription effectively (Jordan and Murphy, 1990; Thompson, *et al.*, 1989; Wakeling, 1992; Wolf and Fuqua, 1995).

Eugenol (**84**) did not demonstrate oestrogenic activity in the yeast screen (refer to 4.7.4, Figure 4.14) but did competitively displace [^{3}H]-17β-oestradiol from ERα and ERβ (refer to 4.8.2 and Figures 4.19, 4.22 and 4.23). These results indicate that eugenol (**84**) may have partial agonist or antagonistic effects for the ER. To investigate this, an anti-oestrogen screen was conducted. This involves evaluating the inhibition of E2 (**14**) activity by a particular chemical (Routledge and Sumpter, 1997; Sohoni and Sumpter, 1998). The oestrogenic effect of E2 (**14**) (0.78nM) in the presence of eugenol (**84**) was assessed in the yeast screen. 0.78nM E2 (**14**) was used, as this concentration allows both positive and negative effects on β-galactosidase activity to be assessed.

In the yeast screen, eugenol (**84**) ($p<0.001$) produced a significant dose-dependent decrease in β-galactosidase activity in the presence of E2 (**14**), which was associated with a decrease in CPRG metabolism (Figure 4.25). This indicates eugenol (**84**) has

weak antagonistic activity (EC_{50}: 231.0μM). The positive control, hydroxytamoxifen ($p<0.001$; EC_{50}: 0.4μM), significantly antagonised E2 (**14**) stimulation of β-galactosidase activity, but was almost 600-fold more potent than eugenol (**84**) (Figure 4.25). Hydroxytamoxifen has previously been reported to inhibit E2 (**14**) induced expression of β-galactosidase activity (Routledge and Sumpter, 1997; Sohoni and Sumpter, 1998).

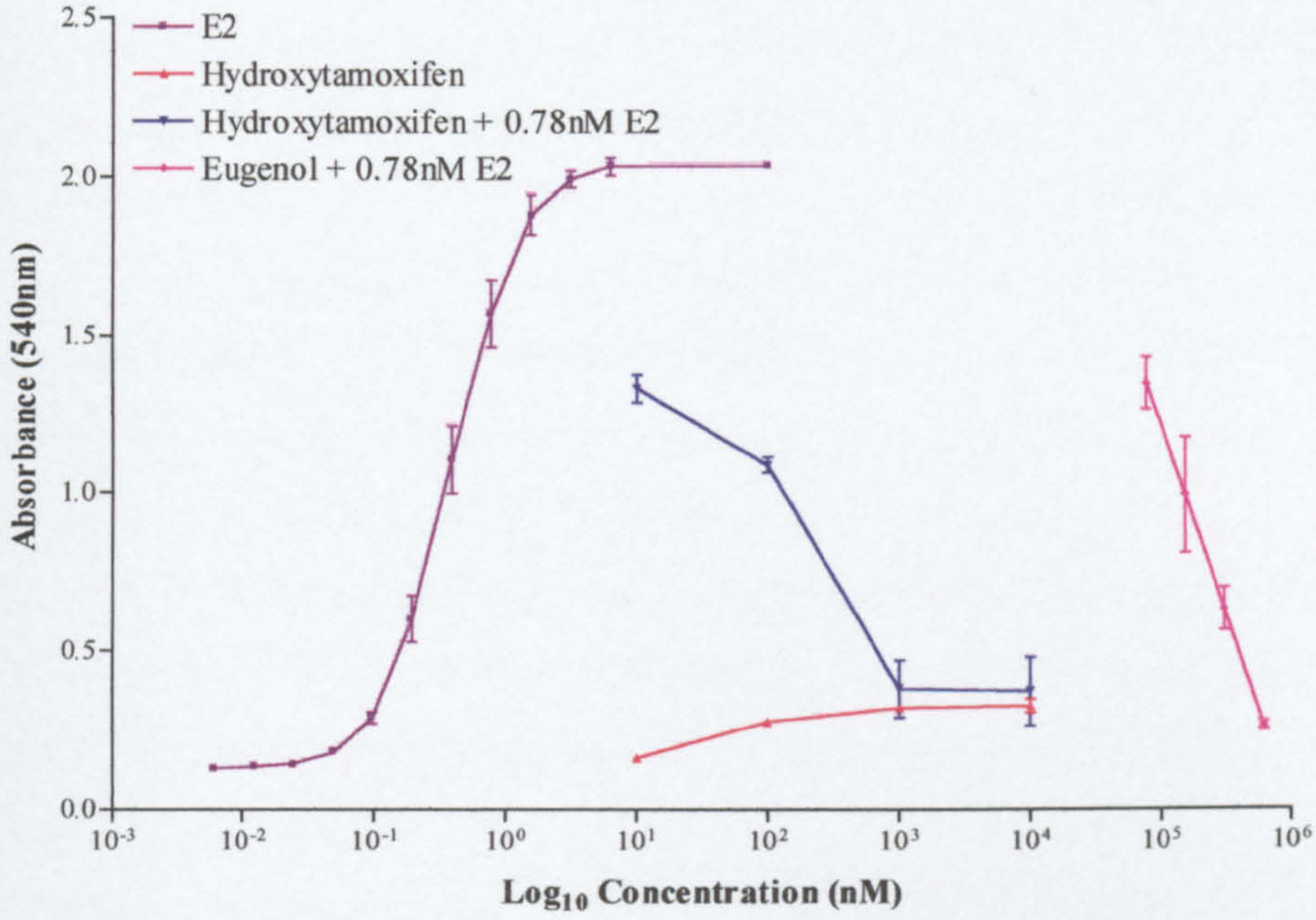


Figure 4.25. Anti-oestrogenic and oestrogenic activity of hydroxytamoxifen ($p<0.001$), hydroxytamoxifen in the presence of 0.78nM 17β-oestradiol ($p<0.001$), eugenol in the presence of 0.78nM 17β-oestradiol ($p<0.001$) and 17β-oestradiol ($p<0.001$), assessed by stimulation of β-galactosidase activity in genetically modified yeast cells (n=3-6 ± SD).

Hydroxytamoxifen appeared to show weak partial agonist activity in the absence of E2 (**14**) (Figure 4.25). This occurrence has previously been reported (Beresford *et al.*, 2000). It has been proposed that the action of tamoxifen is similar to hydroxytamoxifen, and shows weak agonistic activity at low concentrations, but acts antagonistically at higher concentrations (Hedden *et al.*, 1995). The mechanism of action of tamoxifen involves binding to the LBD at low concentrations, thus allowing expression of ligand-independent activation function but, as tamoxifen concentration increases and the LBD becomes saturated, interaction with a second domain on the

ER occurs (which is not recognised by E2 (**14**)), which abolishes the agonistic activity (Hedden *et al.*, 1995; Wolf *et al.*, 1995).

This occurrence may therefore also explain weak agonistic activity of hydroxytamoxifen in the yeast assay in the absence of E2 (**14**), when ER saturation had not occurred. The anti-oestrogens tamoxifen and hydroxytamoxifen have been shown to inhibit E2 (**14**) induced β-galactosidase activity in the yeast screen, but the anti-oestrogen ICI 182, 780 did not inhibit the E2 (**14**) induced response and showed agonistic activity in the yeast screen but ICI 182, 780 did display anti-oestrogenic activity in other bioassays (Beresford *et al.*, 2000; Routledge and Sumpter, 1997; Sohoni and Sumpter, 1998; Zacharewski, 1997). It is therefore apparent that the yeast screen is useful for detection of some anti-oestrogens but not others, perhaps due to different mechanisms of anti-oestrogenic action of the substance being investigated. This occurrence may be explained by the yeast not containing the appropriate proteins necessary for antagonistic activity (Gaido *et al.*, 1997).

The effect of eugenol (**84**) on β-galactosidase activity in the yeast cells, stimulated by E2 (**14**), was also assessed for four days after the three day incubation period of the assay was completed. The results show that β-galactosidase activity increases as a function of time (Figure 4.26).

This is perhaps due to the eugenol (**84**) concentration in the assay decreasing with time, due to its volatility and perhaps metabolism to other compounds (see below for assessment of eugenol metabolism by yeast cells: 4.10.2).

As eugenol (**84**) concentration decreases, so does the antagonistic effect, hence the increased β-galactosidase activity in the presence of 0.78nM E2 (**14**). β-Galactosidase activity also increased as a function of time in the presence of E2 (**84**), absent from antagonists (Figure 4.26). This observation may be explained by the continued metabolism of CPRG to CPR by β-galactosidase in the presence of E2 (**14**).

To determine if the decrease in β-galactosidase activity was due to antagonism of E2 (**14**) by eugenol (**84**), and not due to cytotoxic activity of eugenol (**84**), the number of yeast cells was counted after completion of the three day assay incubation period. The results showed no significant difference ($p>0.05$) between cell number in the presence and absence or eugenol (**84**), nor was cell number significantly affected by eugenol (**84**) concentration, over the concentration range 76.1μM - 609.0μM (Figure 4.27). This suggests eugenol (**84**) may have inhibited the oestrogenic activity of E2 (**14**) by competitive antagonism for the ER.

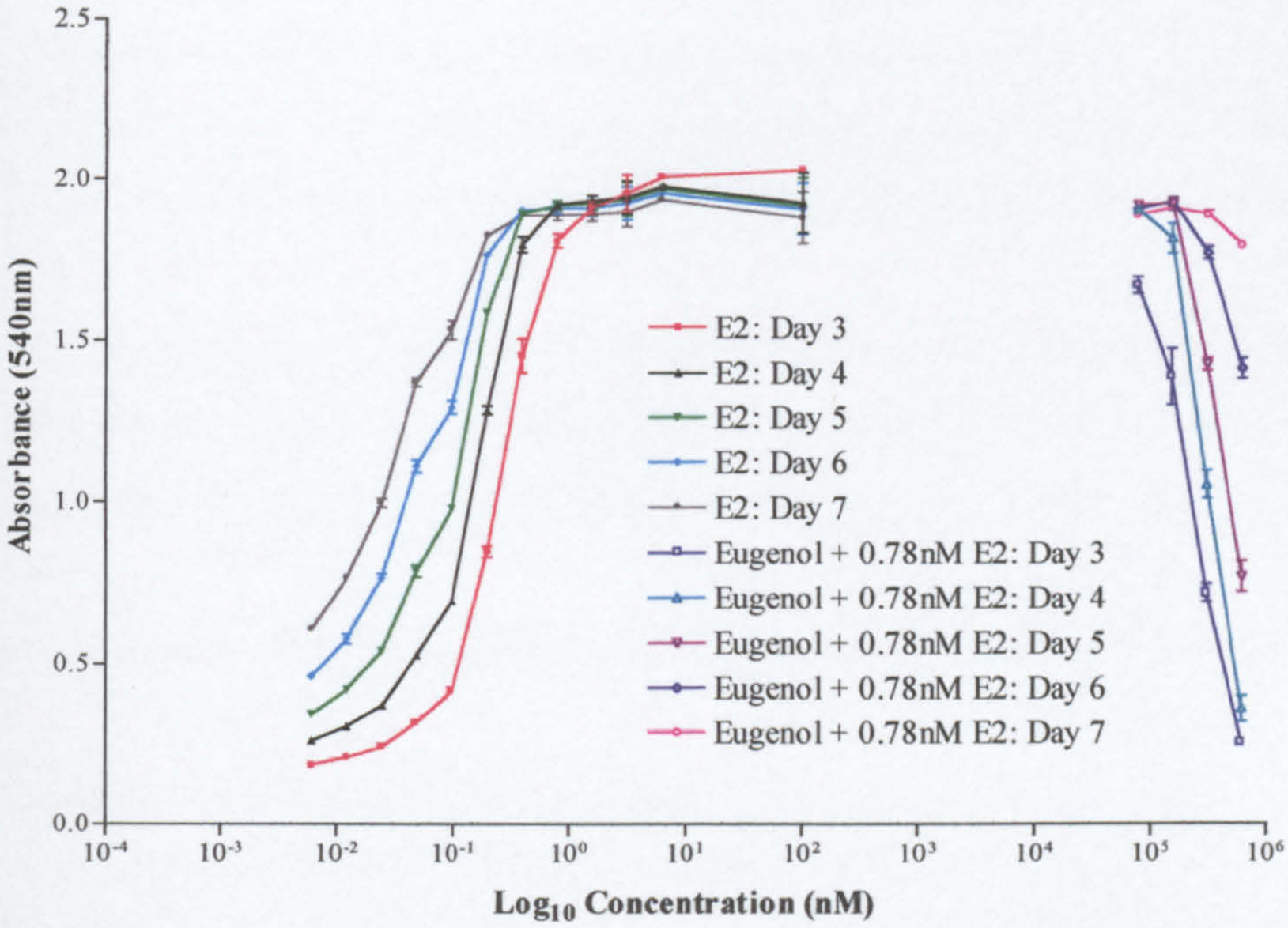


Figure 4.26. Anti-oestrogenic activity of eugenol in the presence of 0.78nM 17β-oestradiol (p<0.001) and oestrogenic activity of 17β-oestradiol (p<0.001), assessed by stimulation of β-galactosidase activity in genetically modified yeast cells, as a function of time (n=3-6 ± SD).

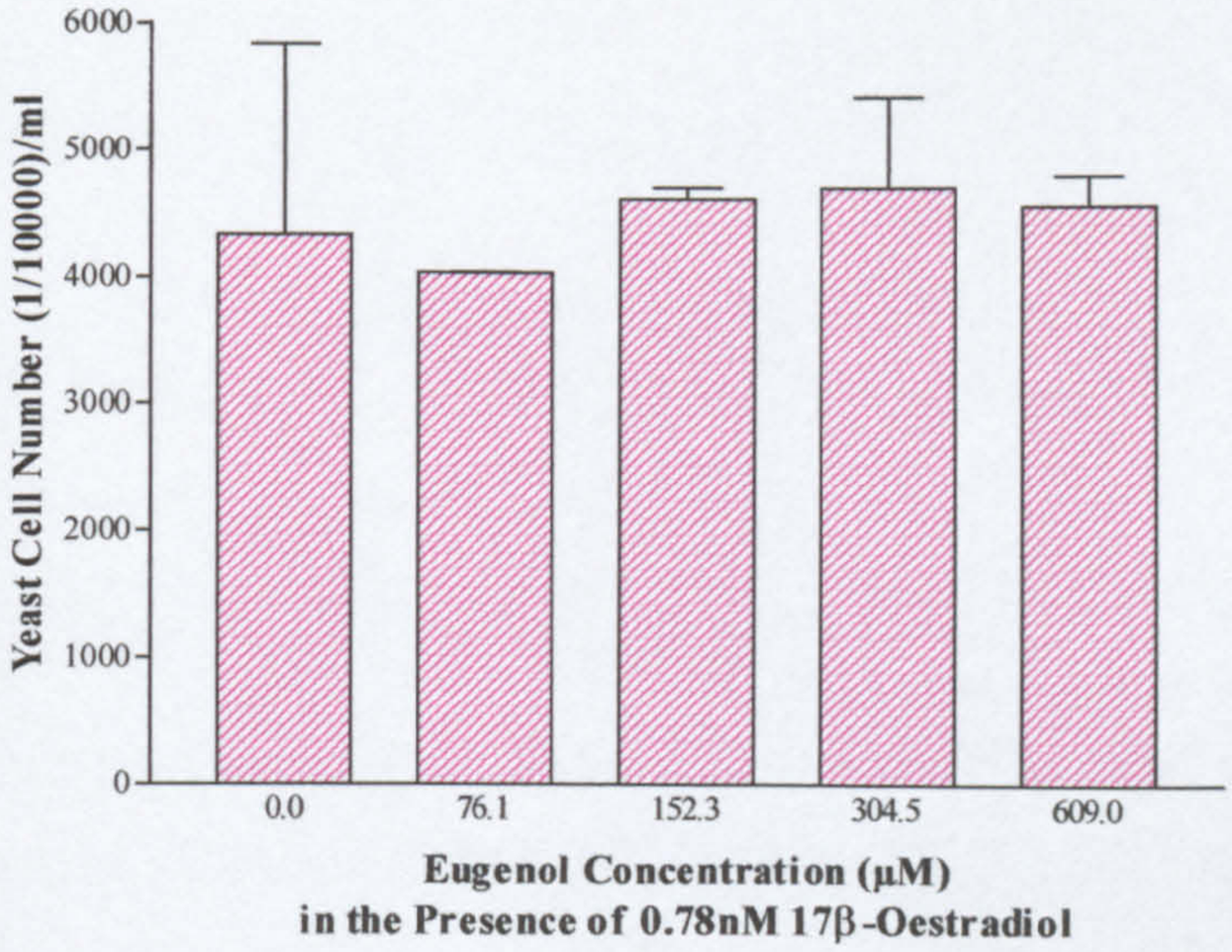


Figure 4.27. Yeast cell number in the presence and absence of eugenol and 0.78nM 17β-oestradiol, after three days incubation at 32°C (n=10 ± SD).

Eugenol (**84**) may have antagonised E2 (**14**) binding to the ER in the yeast screen by competitively binding to the LBD domain of the ER directly and preventing dimerisation, or by preventing the binding of the receptor dimer to ERE, or by mechanisms independent of ER LBD binding, perhaps by interaction with allosteric sites.

Further investigations are required for more complete characterisation of the potential anti-oestrogenic properties of eugenol (**84**). The potential for eugenol (**84**) to interact with the LBD of the ERα was investigated using molecular graphics (refer to 4.11).

4.10.2 Assessment of the Metabolism of Eugenol, in a Recombinant Yeast Screen

To investigate if the apparent lack of oestrogenic effects of eugenol (**84**) in the yeast screen (refer to 4.7.4) were due to the metabolism of eugenol (**84**) to other compounds, or if the apparent antagonistic activity in the yeast screen (refer to 4.10.1) was due to a metabolite, the metabolism of eugenol (**84**) by the yeast cells was assessed. The results show that eugenol (**84**) concentration decreased with time in the presence and absence of yeast indicating loss of eugenol (**84**) from each well due to its volatility (Table 4.8).

Table 4.8. Assessment of metabolism of eugenol by yeast. Compounds detected in the presence and absence (*x*) of yeast. Peak area determined by ITD.

	Peak Area for Compound Detected	
Time (h)	**Eugenol**	**Phenylethyl Alcohol**
0	31.65 (31.95)	0 (0)
1	26.70 (28.90)	0 (0)
3	23.20 (33.95)	0 (0)
24	25.90 (28.20)	0.50 (0)
48	21.10 (27.00)	2.00 (0)
72	17.60 (17.40)	2.10 (0)

Phenylethyl alcohol concentration increased with time in the presence of yeast but was not detected in the absence of yeast, indicating it was a metabolic product of eugenol (**84**). The loss of eugenol (**84**) from the assay medium, shown by the decrease in eugenol (**84**) concentration in the absence of yeast (Table 4.8) presumably explains the loss of the antagonistic effect of eugenol (**84**) with time in the yeast screen (refer to 4.10.1, Figure 4.26).

The potential anti-oestrogenic effects of phenylethyl alcohol remain to be identified, however, as eugenol (**84**) displaced binding of [^{3}H]-17β-oestradiol from isolated ERα and ERβ (refer to 4.8.2) in the absence of the metabolic processes which occurred in the yeast cells, it is more likely that eugenol (**84**) was responsible for the anti-oestrogenic effects observed in the yeast screen (refer to 4.10.1).

4.11 Results and Discussion: Structure-Activity Assessment Using Molecular Graphics: Potential Interactions of Citral (a and b), Eugenol, Geraniol and Nerol with the α-Oestrogen Receptor

4.11.1 Conformational Search Results

This study proposes the agonist binding mechanism of citral a (**70**), citral b (**71**), geraniol (**72**) and nerol (**75**), and the antagonist binding mechanism of eugenol (**84**) for the LBD of the ERα. Torsion angles (TOR) on the molecule under investigation were selected (refer to method, 4.4 and Figure 4.2) prior to a conformational search performed for identification of low energy conformations. The search results generated gave the energy and torsion angles for each conformation of the molecule. Following inspection of the data, similar conformations of the molecule were grouped together (Tables 4.9 - 4.13).

After selecting the conformation of lowest energy representative of each group (conformation 1 for each group in Tables 4.9 - 4.13) the molecule of the selected conformation was manually introduced into the LBD of the ERα, in such a way that it occupied a position similar to the bound ligands E2 (**14**) or RAL (**95**). The position and conformation of each molecule were further adjusted, by refinement of the

potential energy of the system and the final torsion angles of each molecule positioned within the LBD were determined (Table 4.14).

Table 4.9. Low energy conformations of the molecule citral a (geranial), generated by a conformational search.

Conformation	TOR 1 (°)	TOR 2 (°)	TOR 3 (°)	TOR 4 (°)
1	±180	±180	0	±180
2	+85	±180	+1	±180
3	-85	±180	-2	±180
4	±180	±180	-100	±180
5	±180	±180	+100	±180
6	+85	±180	+100	±180
7	-85	±180	-100	±180
8	-85	±180	+100	±180
9	+85	±180	-100	±180
10	±180	±180	+0.14	+0.05
11	+85	±180	-0.2	-0.2
12	-85	±180	+0.2	+0.2
13	±180	±180	+100	+0.3
14	±180	±180	-100	-0.3
15	-85	±180	+100	+0.3
16	+85	±180	-100	-0.3
17	-85	±180	-100	-0.3
18	+85	±180	+100	+0.3

Table 4.10. Low energy conformations of the molecule citral b (neral), generated by a conformational search.

Conformation	TOR 1 (°)	TOR 2 (°)	TOR 3 (°)	TOR 4 (°)
1	±180	±180	0	±180
2	+85	±180	+1	±180
3	-85	±180	-2	±180
4	±180	±180	+100	±180
5	±180	±180	-100	±180
6	+85	±180	+100	±180
7	-85	±180	-100	±180
8	-85	±180	+100	±180
9	+85	±180	-100	±180
10	±180	±180	-0.1	+1
11	-85	±180	-0.1	+0.1
12	+85	±180	-0.2	-0.1
13	±180	±180	-100	-0.4
14	±180	±180	+100	+0.3
15	-85	±180	+100	+0.3
16	+85	±180	-100	-0.1
17	-85	±180	-100	-0.4
18	+85	±180	+100	+0.3

Table 4.11. Low energy conformations of the molecule eugenol, generated by a conformational search.

Conformation	TOR 1 (°)	TOR 2 (°)	TOR 3 (°)
1	-92.1	96.1	-73.0
2	±180	±180	±180

Table 4.12. Low energy conformations of the molecule geraniol, generated by a conformational search.

Conformation	TOR 1 (°)	TOR 2 (°)	TOR 3 (°)	TOR 4 (°)
1	±180	±180	-55	+110
2	±180	±180	+55	-110
3	±180	±180	-55	-110
4	±180	±180	+55	+110
5	±180	-80	+55	-110
6	±180	+80	+55	-110
7	±180	-80	-55	+110
8	±180	±180	±180	-150
9	±180	+80	-55	+110
10	±180	+80	-55	-110
11	±180	+80	+55	+110
12	±180	-80	-55	-110
13	±180	±180	±180	+130
14	±180	-80	+55	±180
15	±180	±180	±180	±180
16	±180	-80	-55	-155
17	±180	-80	±180	+120
18	±180	+80	±180	-120
19	±180	+80	±180	±180
20	±180	-80	±180	-140
21	±180	0	±180	±180

Table 4.13. Low energy conformations of the molecule nerol, generated by a conformational search.

Conformation	TOR 1 (°)	TOR 2 (°)	TOR 3 (°)	TOR 4 (°)
1	±180	±180	-55	+110
2	±180	±180	+55	-110
3	±180	±180	-55	-110
4	±180	±180	+55	+110
5	±180	-80	+55	-110
6	±180	+80	+55	-110
7	±180	-80	-55	+110
8	±180	±180	±180	-150
9	±180	+80	-55	+110
10	±180	-80	-55	-110
11	±180	+80	+55	+110
12	±180	+80	-55	-110
13	±180	±180	±180	+130
14	±180	-80	+55	±180
15	±180	±180	±180	±180
16	±180	-80	-55	-150
17	±180	+80	±180	-120
18	±180	-80	±180	+120
19	±180	+80	±180	±180
20	±180	-80	±180	-135
21	±180	0	±180	±180

4.11.2 Comparison of the Chemical Structures of the Monoterpenes and Eugenol with 17β-Oestradiol and Raloxifene

Table 4.14. Torsion angles for each molecule complexed with ERα.

Compound	TOR 1 (°)	TOR 2 (°)	TOR 3 (°)	TOR 4 (°)
Citral a (geranial)	+180.0	+179.6	+102.5	+179.9
Citral b (neral)	-85.0	+179.6	+102.5	+179.9
Eugenol	-0.1	-94.0	-114.0	-
Geraniol	-177.4	-68.0	-61.6	-137.6
Nerol	171.3	44.2	161.3	161.1

Each molecule (torsion angles as described in Table 4.14) was analysed to determine any similarities in structure with the endogenous ER agonist E2 (**14**) (and for eugenol (**84**), also with the ER antagonist RAL (**95**)). Each image of a molecule with E2 (**14**) or RAL (**95**) is shown from 3 different angles (the molecule being analysed is represented in bold type) (Figures 4.28 - 4.33).

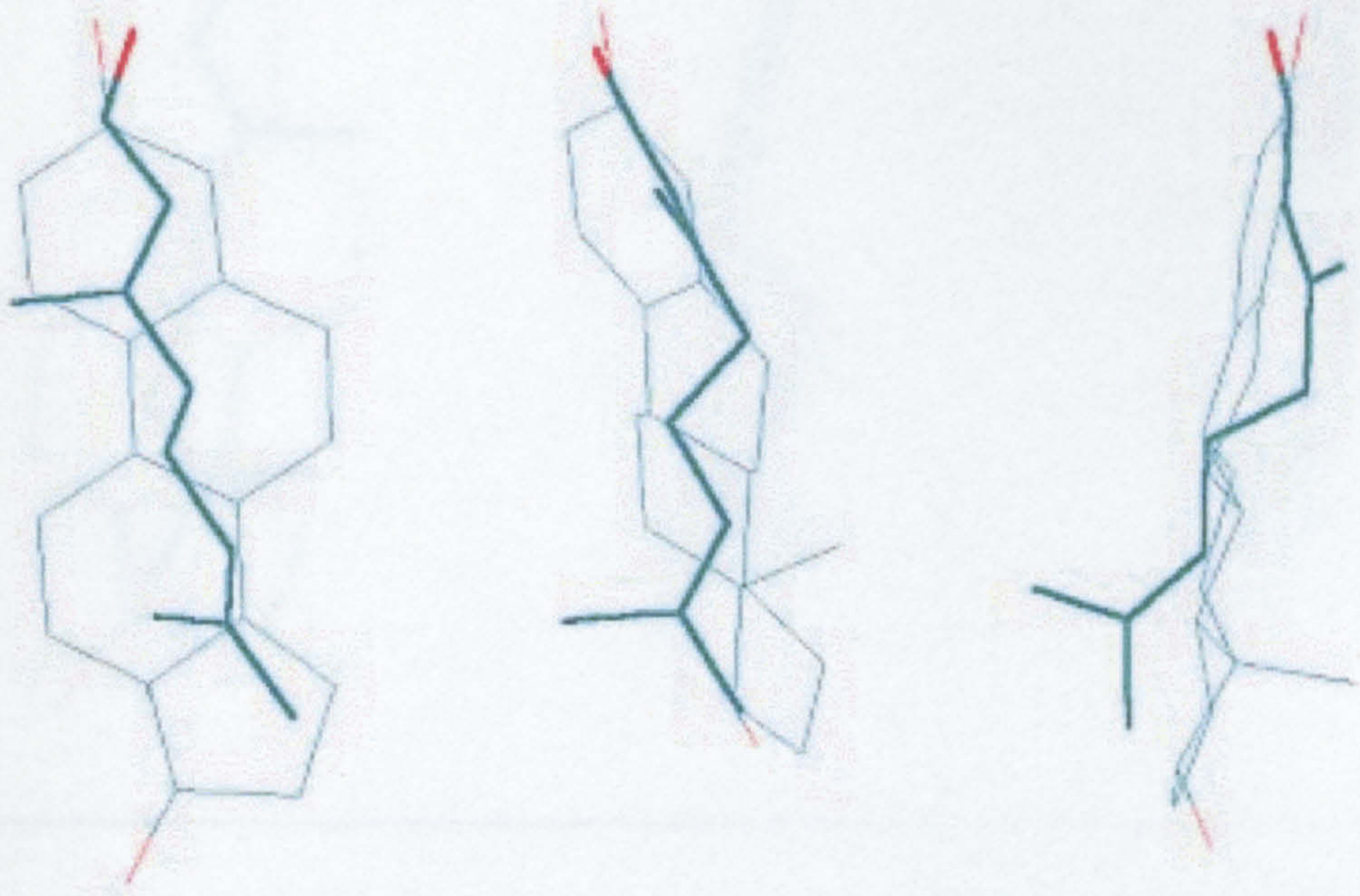


Figure 4.28. Comparison of the structure of citral a (geranial) with 17β-oestradiol, each represented by the proposed conformation in ERα.

The selected conformations of citral a and b (geranial (**70**) and neral (**71**)) show some similarities to the conformation of E2 (**14**) (Figures 4.28 and 4.29). The hydroxyl substituent on the 6-membered ring of E2 (**14**) is similarly positioned to the aldehyde substituent on the citral a (**70**) and b (**71**) molecules. This indicates that both citral a (**70**) and b (**71**) may interact similarly to E2 (**14**) in the LBD of the ER. The planarity of both citral a (**70**) and b (**71**) molecules are also similar to E2 (**14**), although citral a (**70**) is less so than citral b (**71**) (Figures 4.28 and 4.29). There are clear differences between E2 (**14**) and the monoterpene molecules. Citral a (**70**), citral b (**71**), geraniol (**72**) and nerol (**75**) are smaller molecules than E2 (**14**) and would therefore occupy a smaller volume in the LBD. These monoterpenes also lack a second the hydroxyl substituent that is present on the pentacyclic ring of E2 (**14**), and they are also acyclic compounds. However, the configurations of the monoterpenes are possibly similar enough to E2 (**14**), to enable similar interactions in the LBD of the ER.

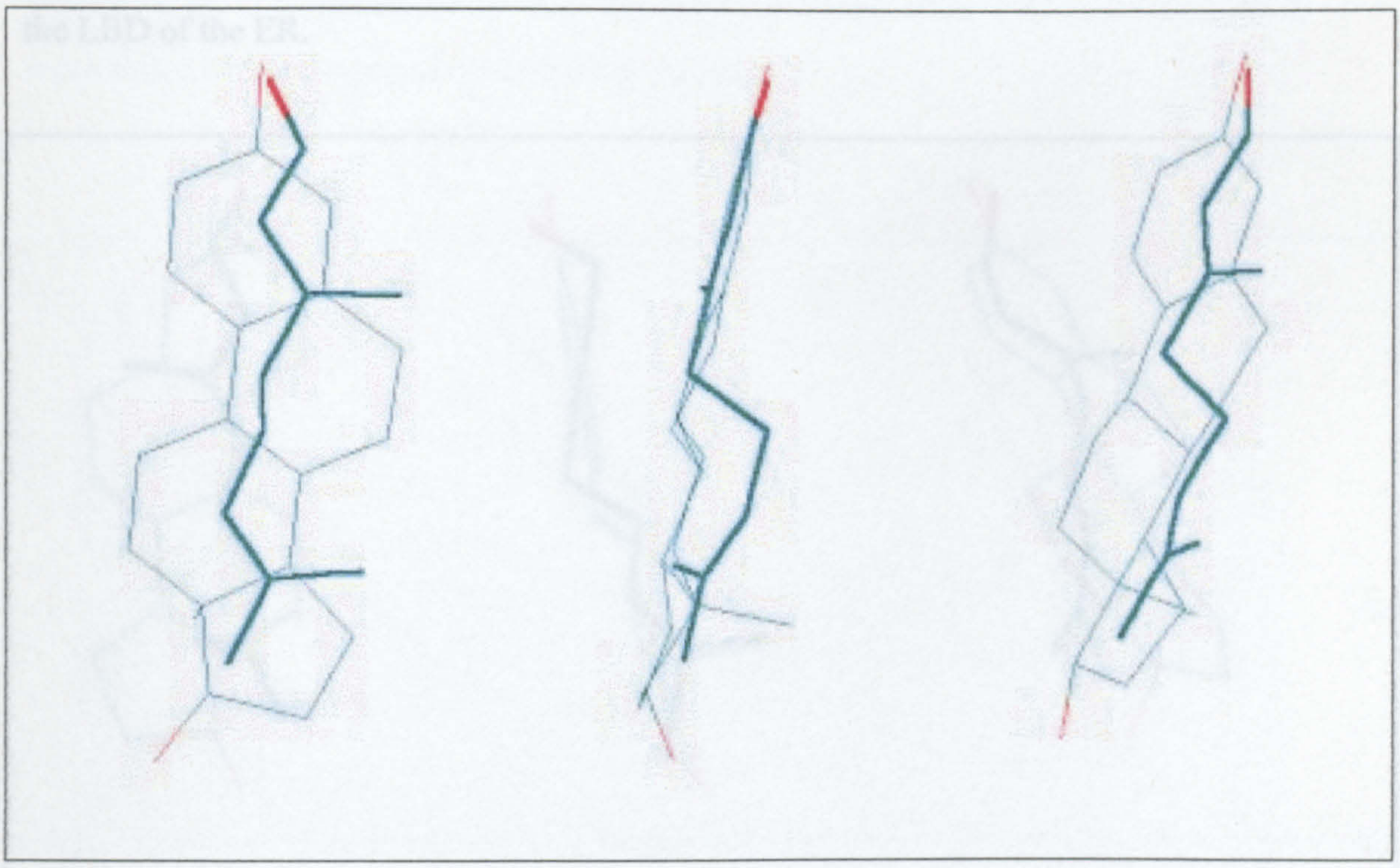


Figure 4.29. Comparison of the structure of citral b (neral) with 17β-oestradiol, each represented by the proposed conformation in ERα.

The configurations of the molecules geraniol (**72**) and nerol (**75**) also enable their hydroxyl substituents to be similarly situated to the hydroxyl substituent on the 6-membered ring of E2 (**14**) (Figures 4.30 and 4.31). The configurations of these monoterpenes also appear to share similar planarity to the E2 (**14**) molecule. The monoterpenes are also considerably hydrophobic, which may enable similar hydrophobic interactions to E2 (**14**), to occur in the LBD. The potential interactions of the monoterpenes with the LBD of ERα were investigated further (Figures 4.35 - 4.38).

The configuration of the molecule eugenol (**84**) showed planarity similar to E2 (**14**) (Figure 4.32), and the phenolic component of eugenol (**84**) may be similarly positioned to the phenolic component of E2 (**14**) in the LBD of the ER, which may explain the relatively high affinity of eugenol (**84**) for ERα, compared to the monoterpenes (refer to 4.8.2). The phenolic moiety of eugenol (**84**) could also be positioned similarly to the phenolic component of RAL (**95**) (Figure 4.33), but eugenol (**84**) is a smaller molecule than RAL (**95**), so may occupy a smaller volume in the LBD of the ER.

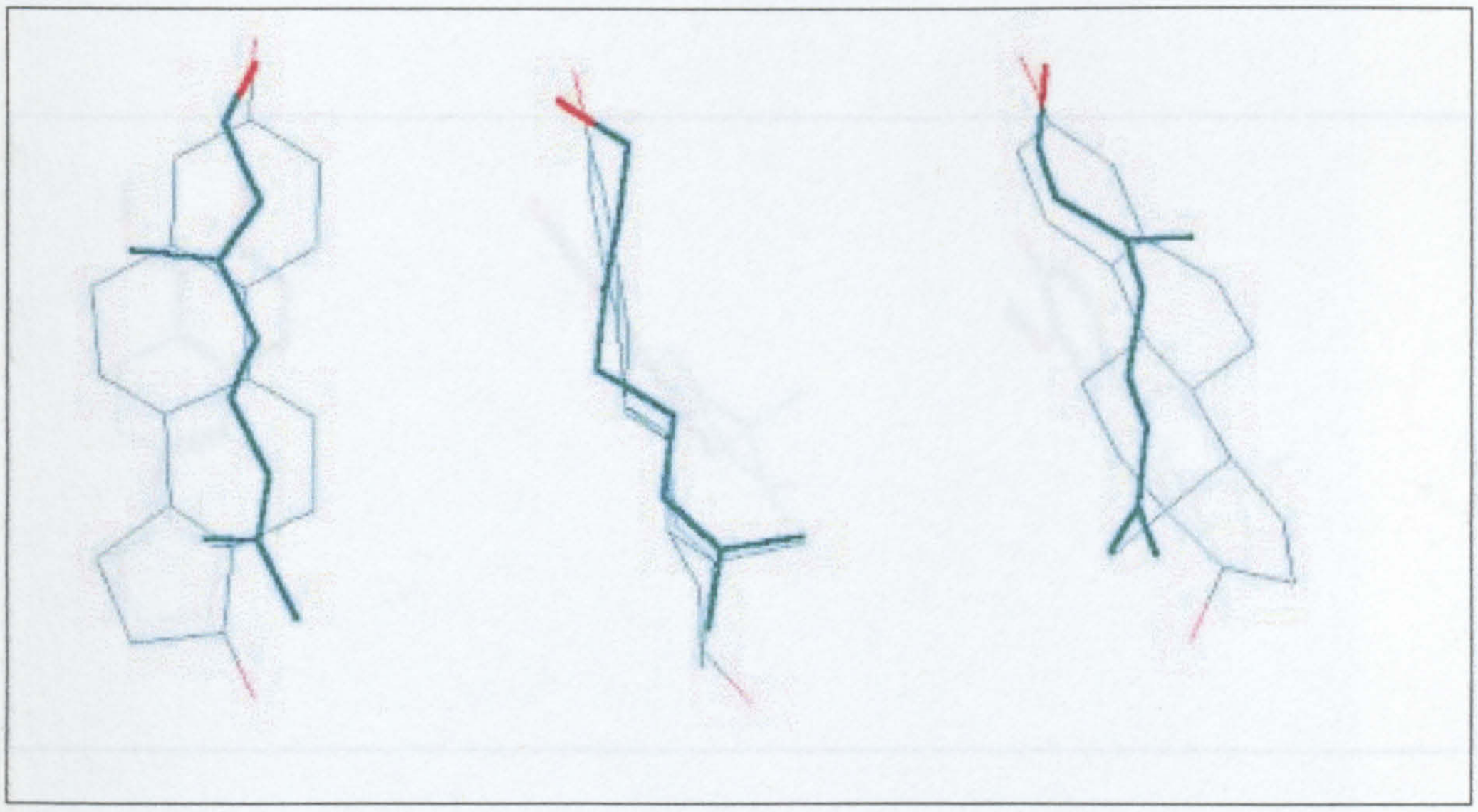


Figure 4.30. Comparison of the structure of geraniol with 17β-oestradiol, each represented by the proposed conformation in ERα.

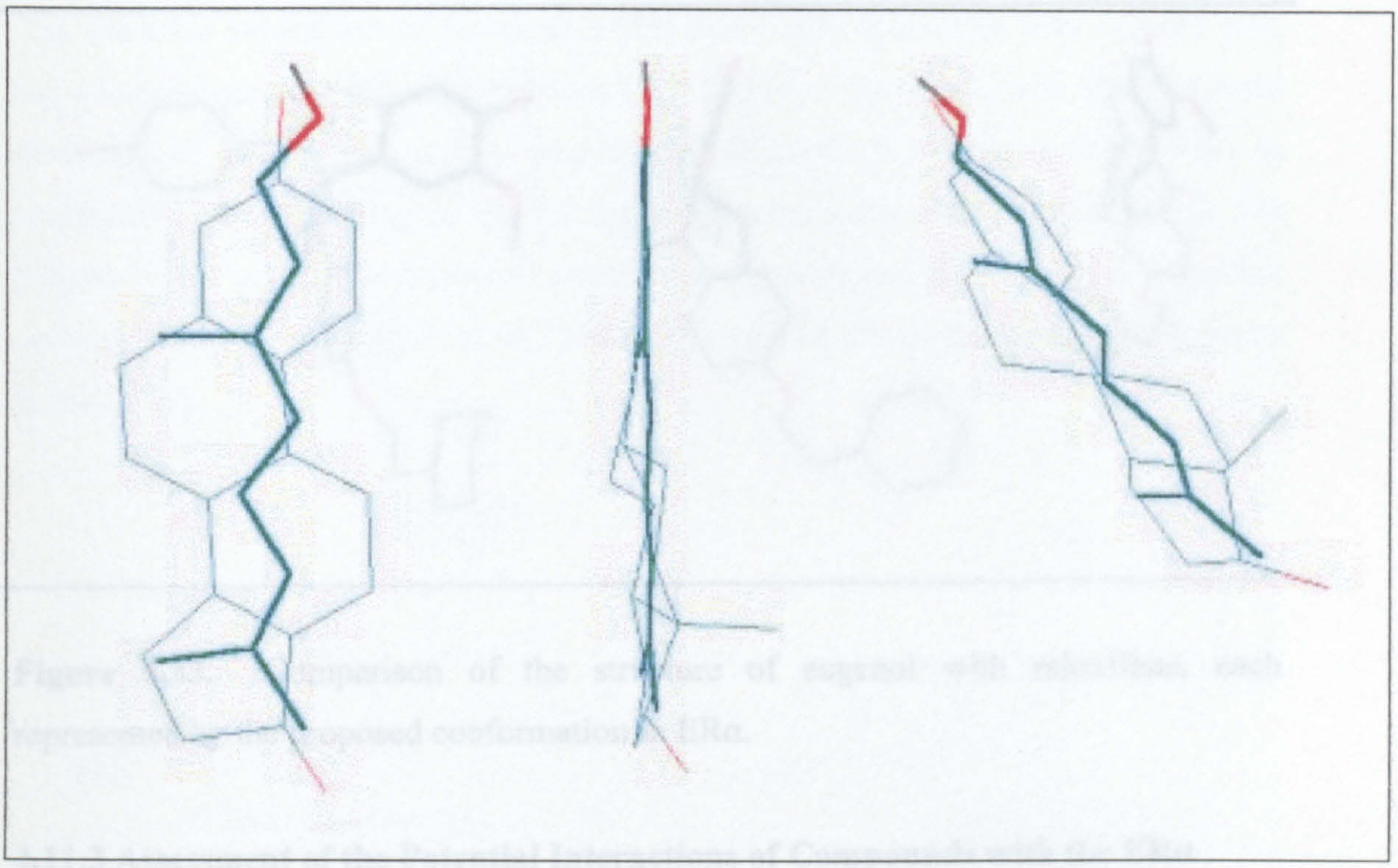


Figure 4.31. Comparison of the structure of nerol with 17β-oestradiol, each represented by the proposed conformation in ERα.

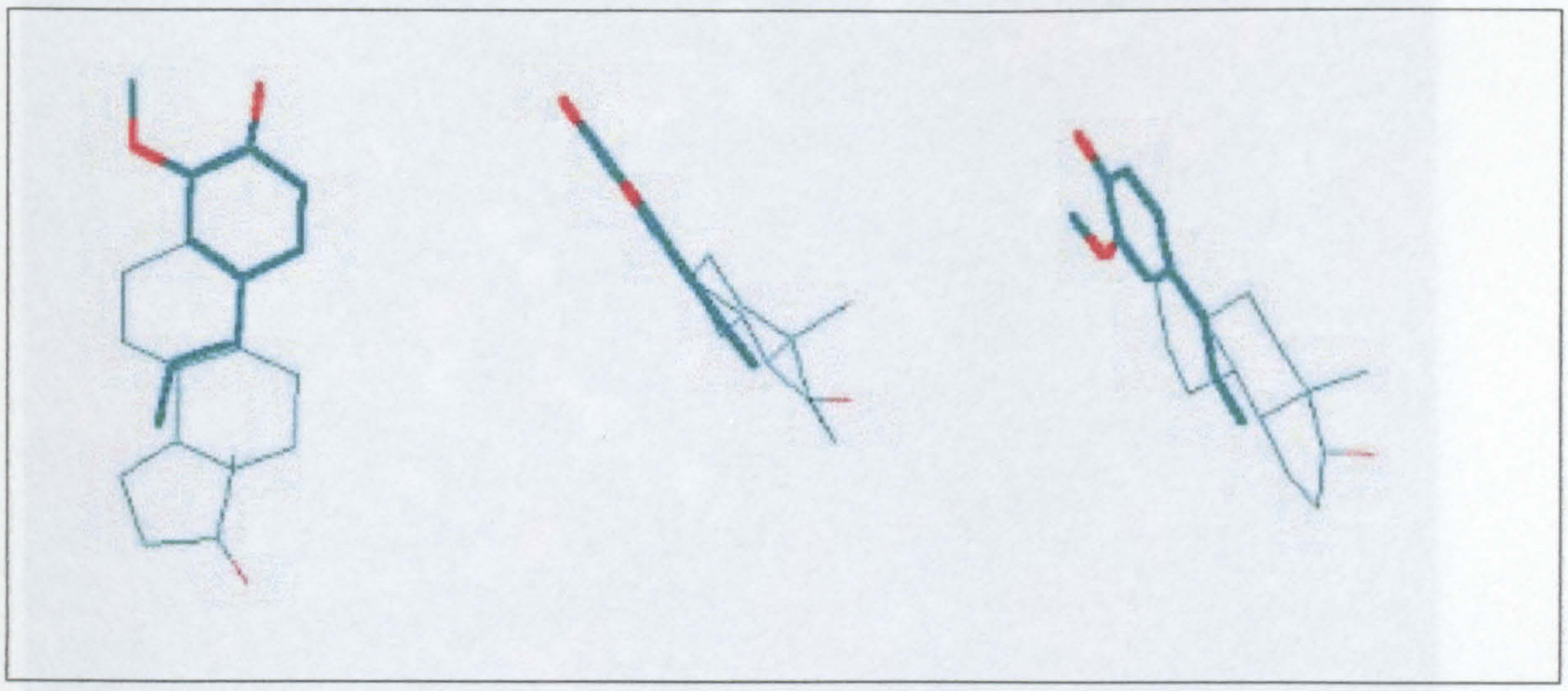


Figure 4.32. Comparison of the structure of eugenol with 17β-oestradiol, each represented by the proposed conformation in ERα.

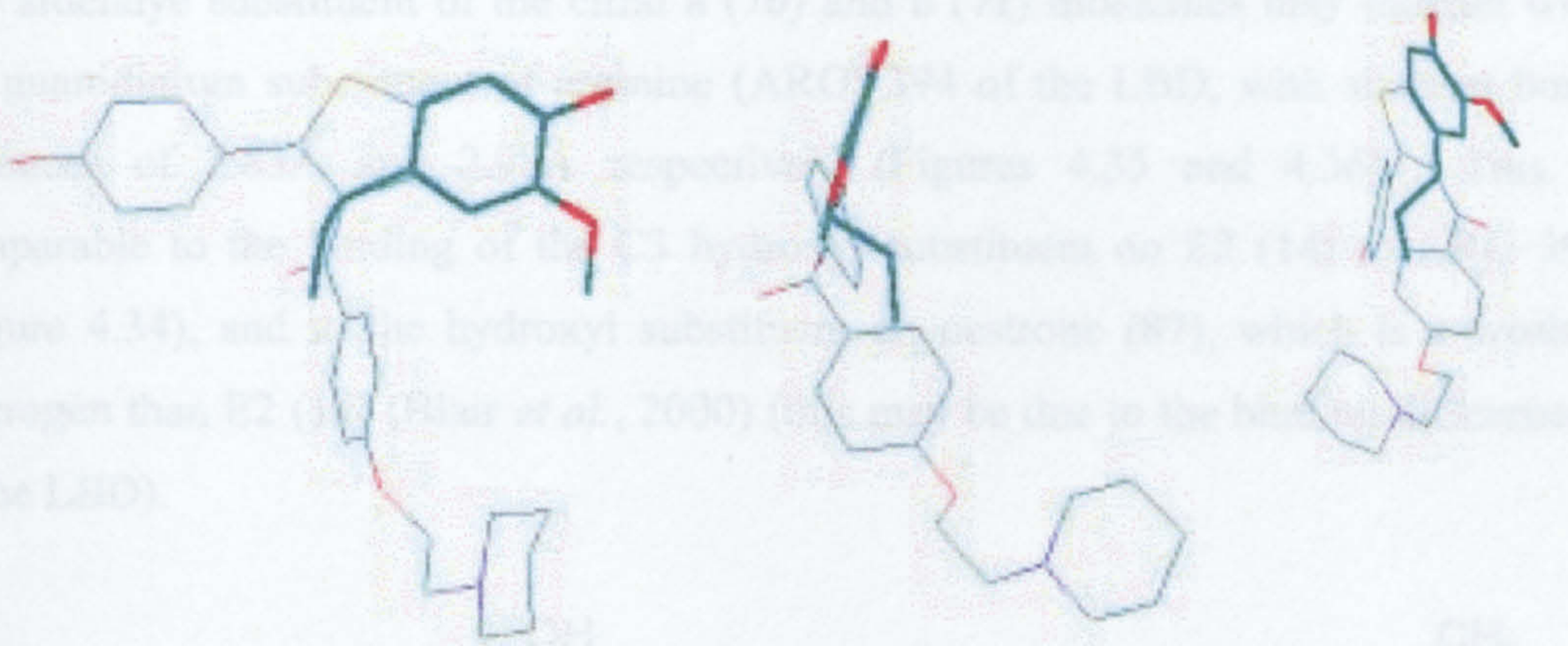


Figure 4.33. Comparison of the structure of eugenol with raloxifene, each represented by the proposed conformation in ERα.

4.11.3 Assessment of the Potential Interactions of Compounds with the ERα

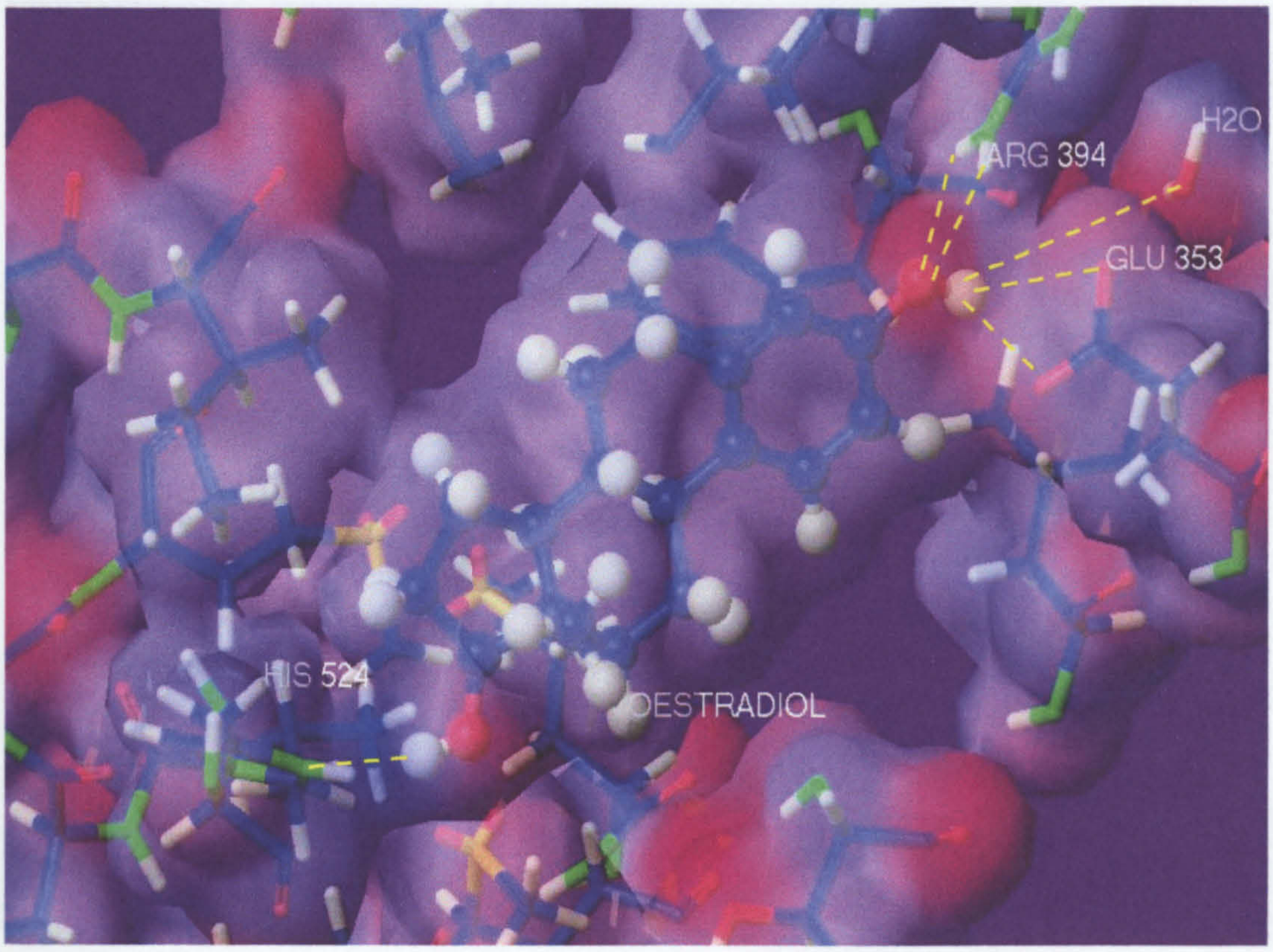
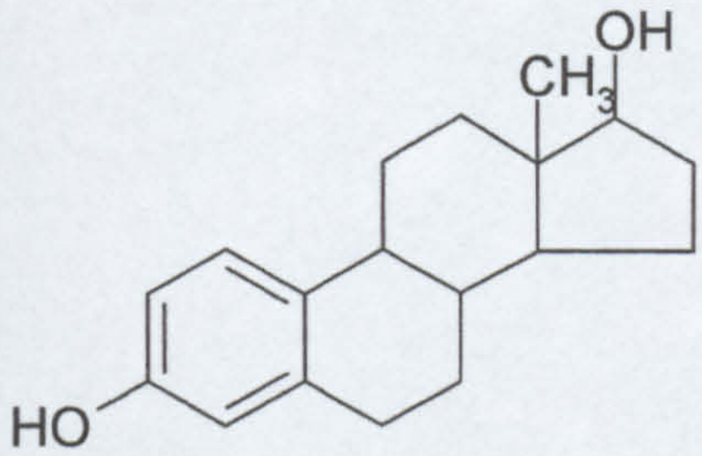
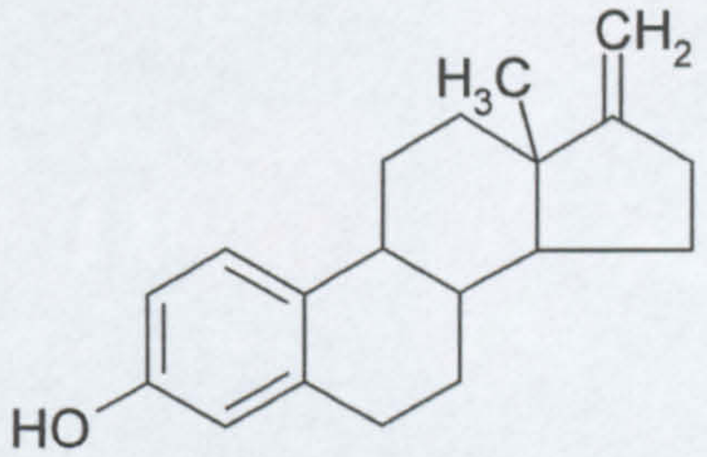


Figure 4.34. Potential interactions (- - - - - -) of 17β-oestradiol with the surrounding residues of the ligand binding domain of the ERα.

The aldehdye substituent of the citral a (**70**) and b (**71**) molecules may interact with the guanidinium substituent of arginine (ARG) 394 of the LBD, with shortest bond distances of 2.83Å and 2.98Å respectively (Figures 4.35 and 4.36). This is comparable to the binding of the C3 hydroxyl substituent on E2 (**14**) to ARG 394 (Figure 4.34), and to the hydroxyl substituent on oestrone (**87**), which is a weaker oestrogen than E2 (**14**) (Blair *et al.*, 2000) (this may be due to the binding differences in the LBD).



17β-Oestradiol (**14**)



Oestrone (**87**)

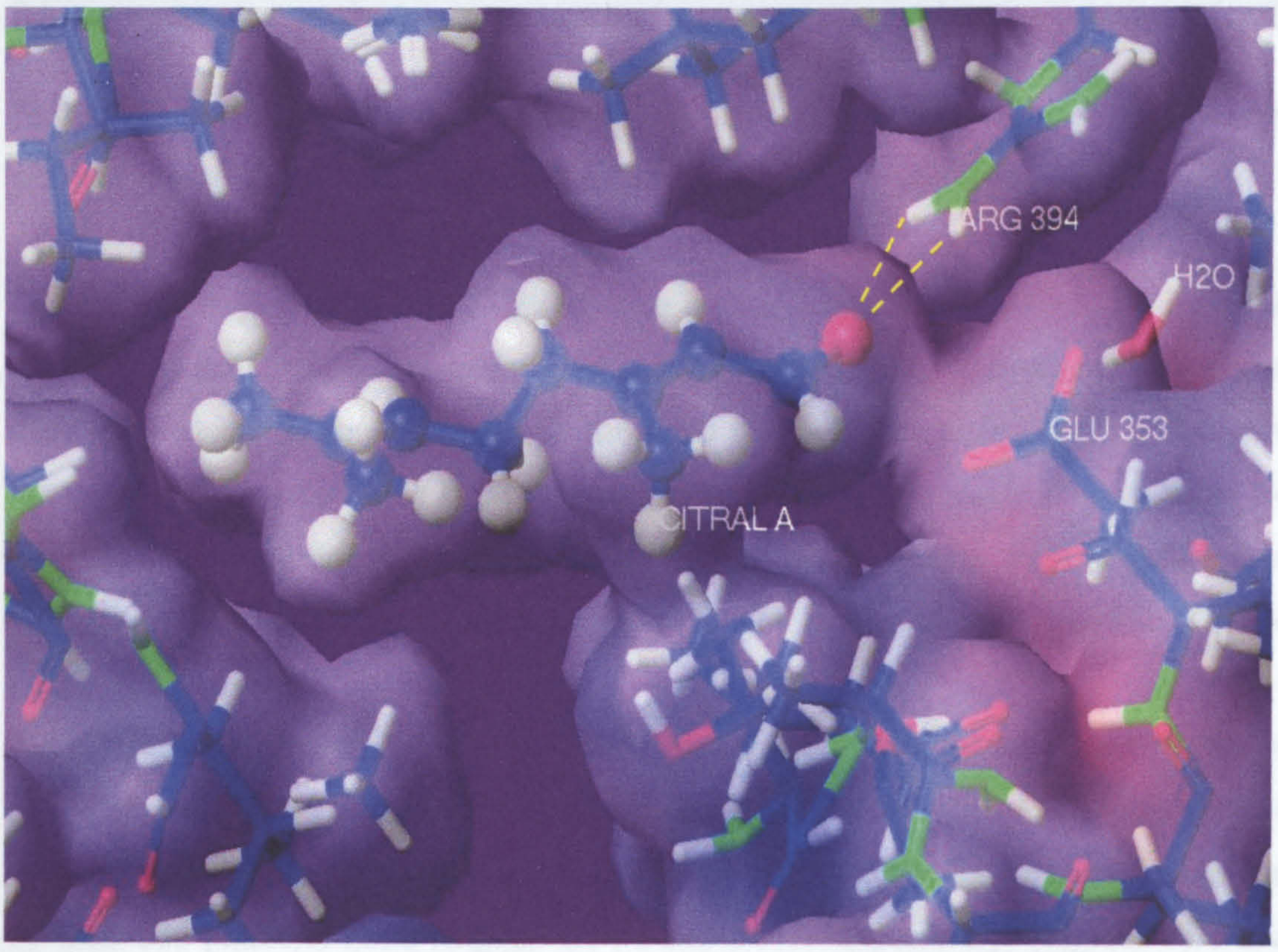


Figure 4.35. Potential interactions (- - - - -) of geranial (citral a) with the surrounding residues of the ligand binding domain of the ERα.

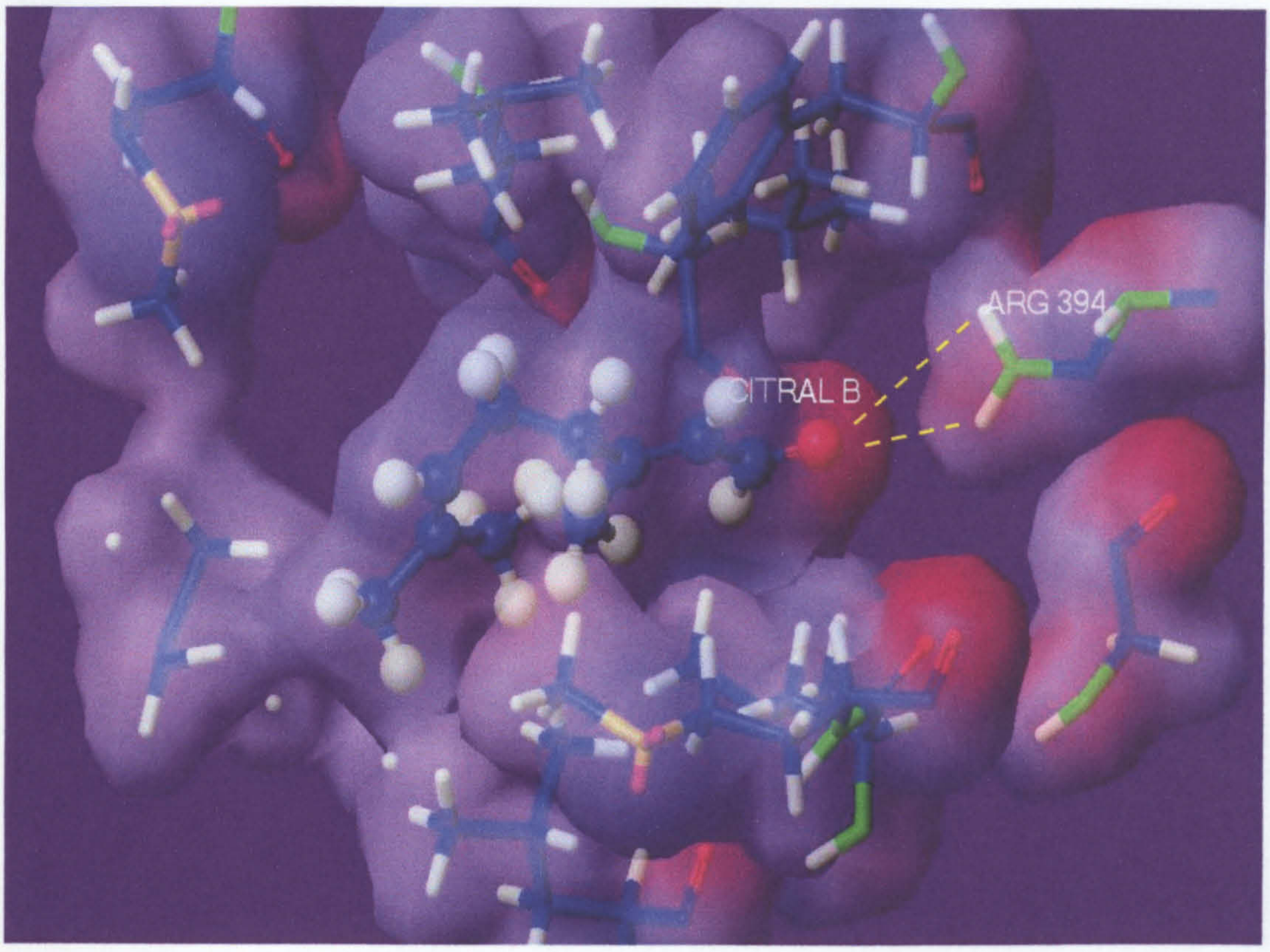


Figure 4.36. Potential interactions (------) of neral (citral b) with the surrounding residues of the ligand binding domain of the ERα.

The C3 hydroxyl substituent of E2 (**14**) also interacts with the carboxylate substituent of glutamic acid (GLU) 353 and a water molecule, and the C17 hydroxyl substituent interacts with histidine (HIS) 524 in the LBD (Brzozowski *et al.*, 1997 and Figure 4.34). The absence of these latter interactions for citral a (**70**) and b (**71**), and also for geraniol (**72**) and nerol (**75**) in the LBD, may reflect the much weaker oestrogenic activity and ER binding properties observed in the *in vitro* assays (refer to 4.7 and 4.8).

Using molecular graphics, it can be shown that the hydroxyl substituents of geraniol (**72**) and nerol (**75**) may interact with both the guanidinium substituent of ARG 394 (shortest bond lengths: 2.06Å and 2.11Å respectively) and the carboxylate substituent of GLU 353 (shortest bond lengths: 2.54Å and 2.80Å respectively) (Figures 4.37 and 4.38).

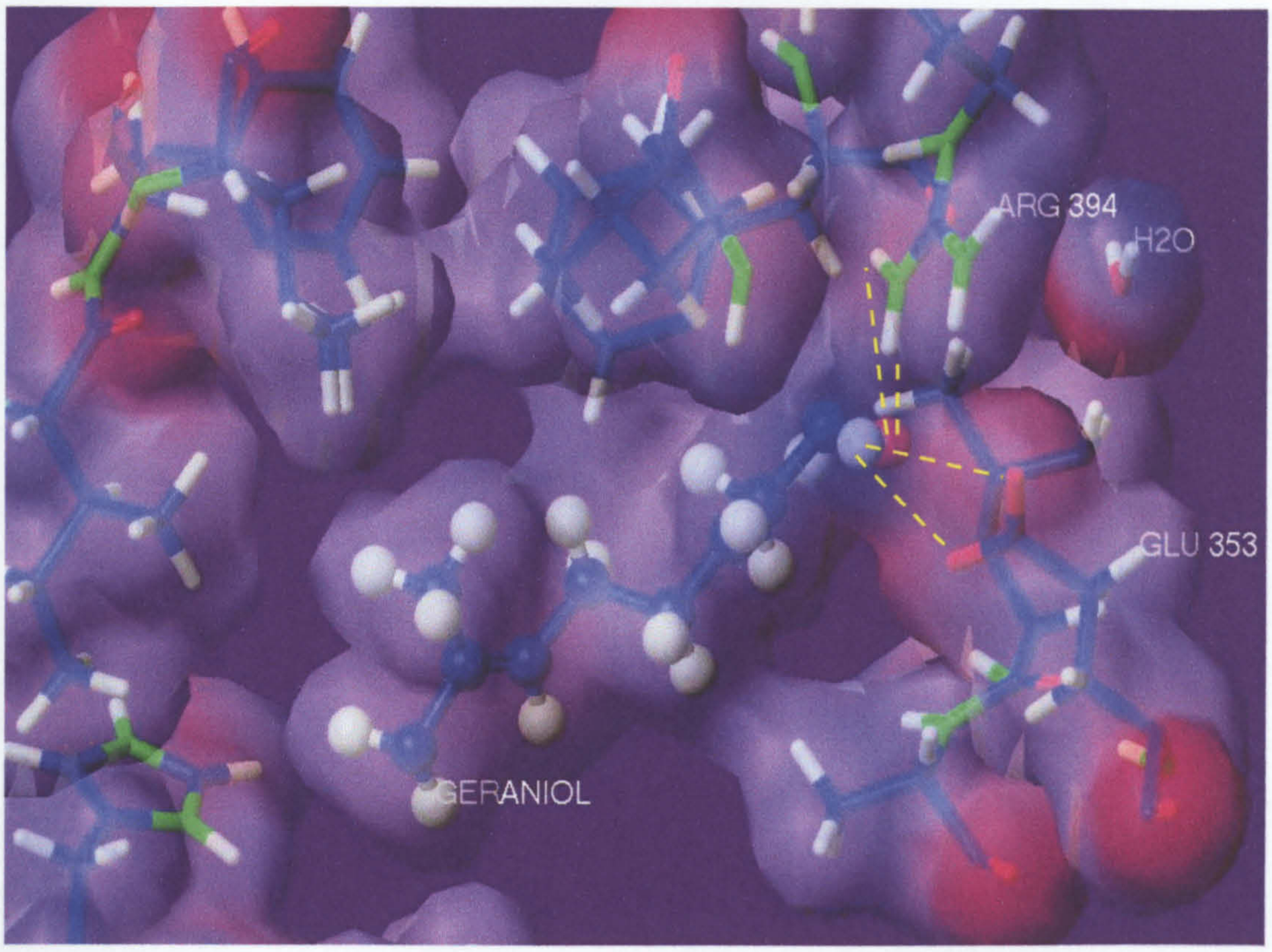


Figure 4.37. Potential interactions (- - - - -) of geraniol with the surrounding residues of the ligand binding domain of the ERα.

E2 (**14**) also participates in a number of hydrophobic interactions in the LBD (Brzozowski *et al.*, 1997 and Figure 4.34). The positioning of the proposed agonist monoterpene molecules may also favourably participate in such hydrophobic interaction, as shown by the atomic van der Waals surfaces of the molecular systems, which show steric complementarity (Figures 4.35 - 4.38).

The structural requirements of a molecule for binding to the hER are complex, however it is generally accepted that a cyclic component (often phenolic) is a structural requirement, as is a substituent (C3 hydroxyl for E2 (**14**)) acting primarily as a hydrogen bond donor in the LBD of hER (Blair *et al.*, 2000; Brzozowski *et al.*, 1997; Dodge, 1998; Katzenellenbogen, 1995; Miller *et al.*, 2001; Mueller and Kim, 1978; Routledge and Sumpter, 1997). Therefore, the acyclic monoterpenes (citral a (**70**), citral b (**71**), geraniol (**72**) and nerol (**75**)) are novel as potential ERα ligands.

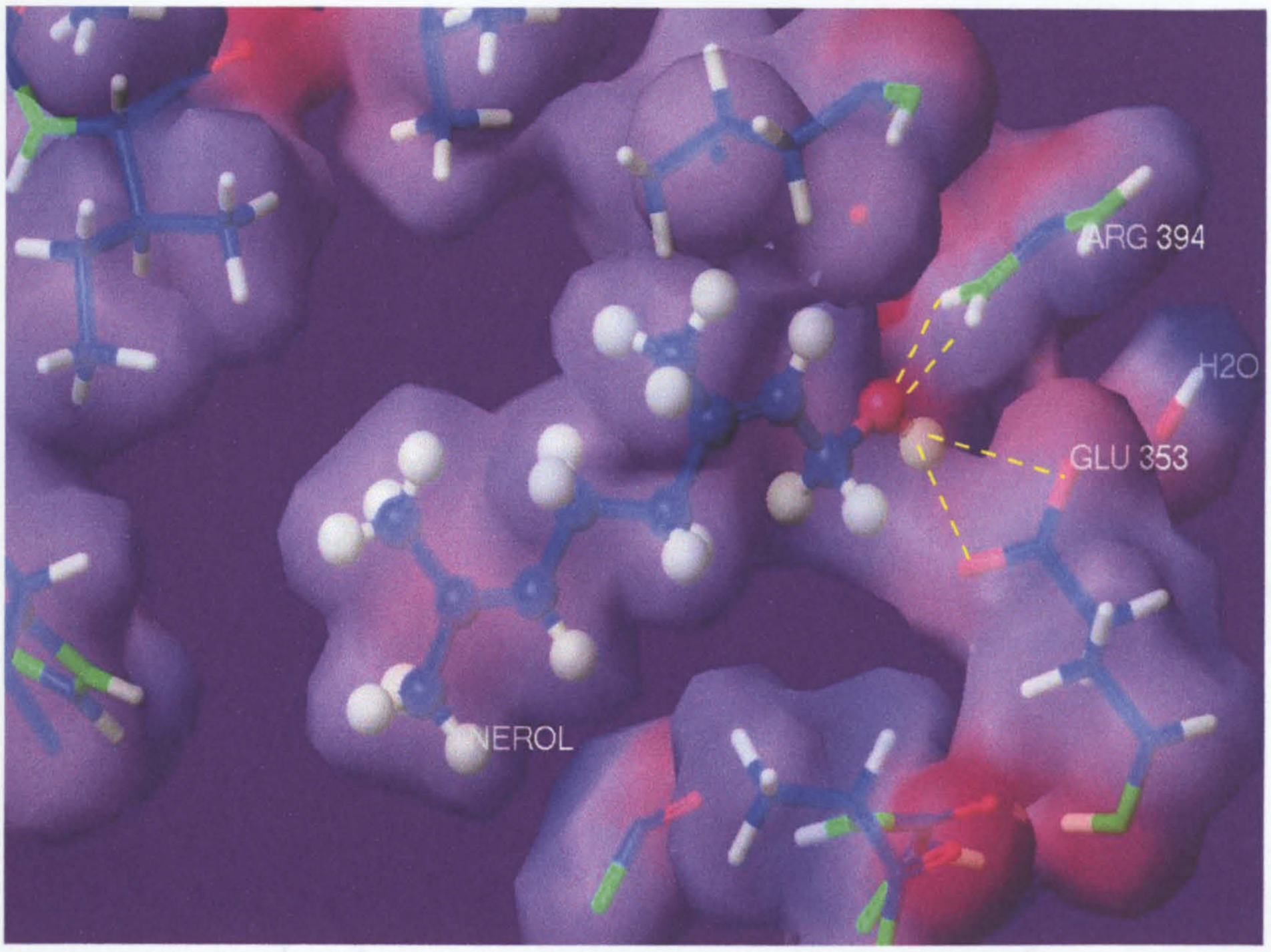
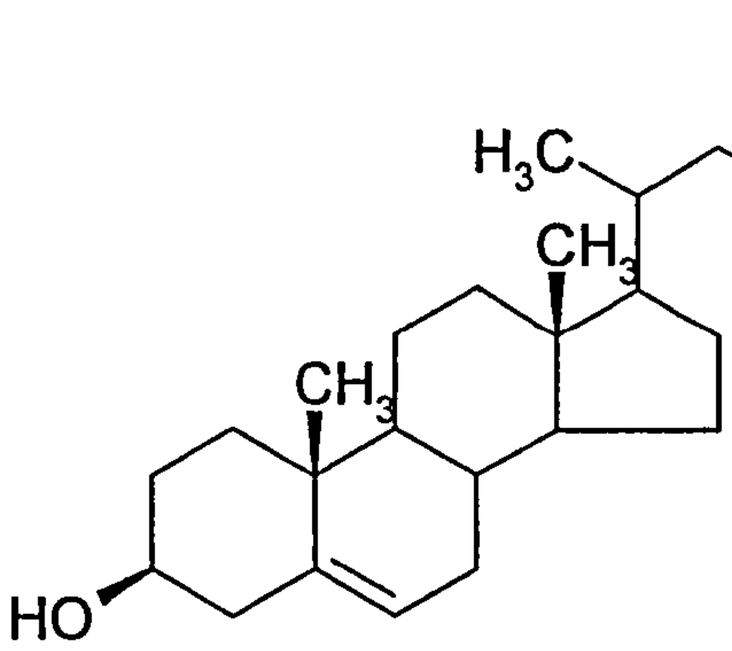


Figure 4.38. Potential interactions (- - - - -) of nerol with the surrounding residues of the ligand binding domain of the ERα.

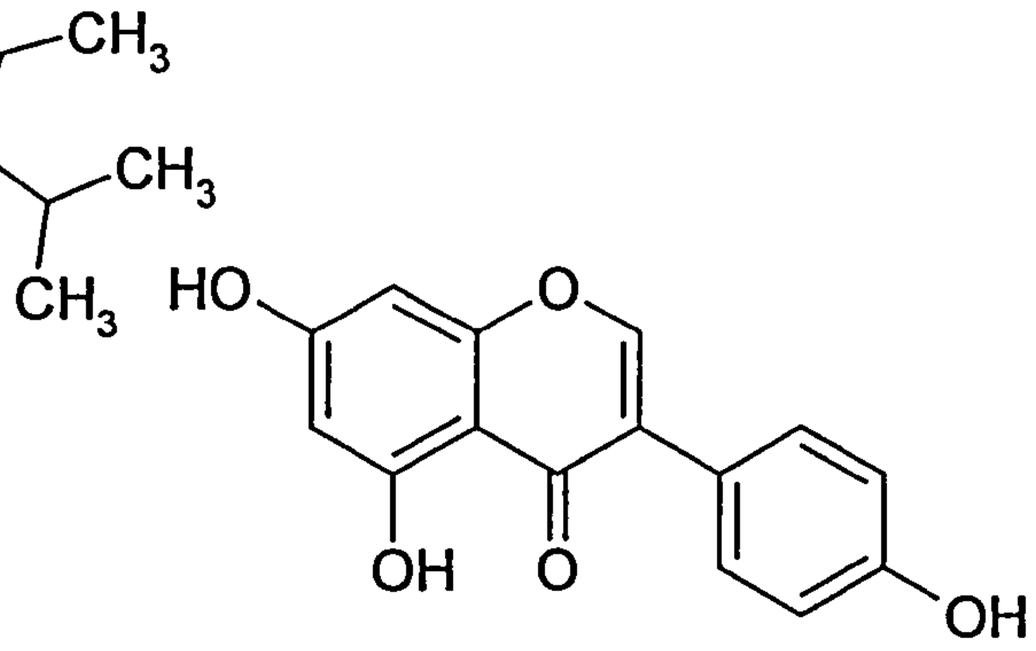
To date, a vast array of ligands, which have affinity for the hER, have been identified, and include the endogenous oestrogens (e.g. E2 (**14**)), which are steroidal, and non-steroidal compounds (e.g. alkyl phenols) (Blair *et al.*, 2000; Dodge, 1998; Harris *et al.*, 1997; Miller *et al.*, 2001; Mueller and Kim, 1978; Routledge *et al.*, 1998; Routledge and Sumpter, 1996; Routledge and Sumpter, 1997).

E2 (**14**) has a hydrocarbon core structure, with a phenolic substituent at the C3 position of the aromatic A-ring, and an alcohol substituent at the C17 position. Other natural steroids may have a ketone group at the C17 position, for example, oestrone (**87**). This may reflect its considerably weaker oestrogenic activity than E2 (**14**). Both E2 (**14**) and oestrone (**87**) have been identified in plant tissue (Harborne and Baxter, 1993), which is surprising as phytosterols generally differ from animal sterols. The structure of phytosterols often consists of an additional methyl (or ethyl) substituent in the side chain (e.g. sitosterol (**88**)). E2 (**14**) is reported to occur in the seeds of the *Phaseolus vulgaris*, and oestrone (**87**), in seeds of both *Phoenix dactylifera* and *Punica granatum* (Harborne and Baxter, 1993). Therefore, it would

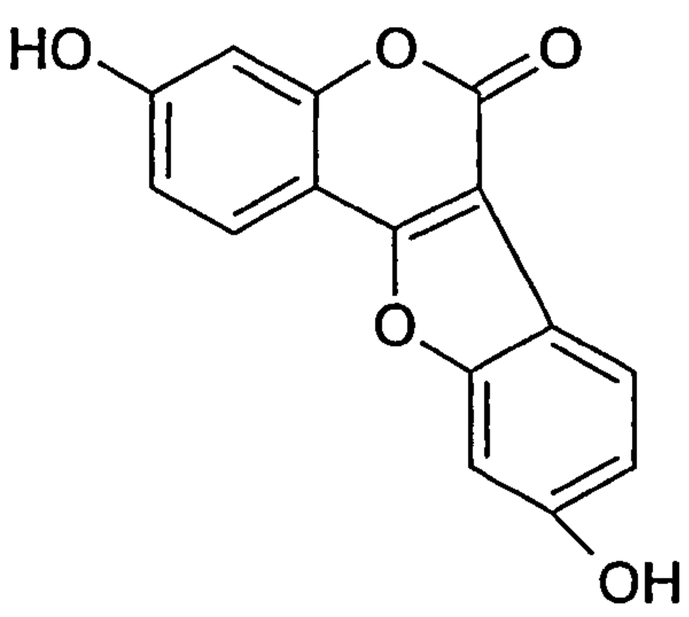
not be surprising to discover that other groups of compounds (synthesised by plants), such as the monoterpenoids and phenylpropanoids, can interact with steroidal receptors, such as hER.



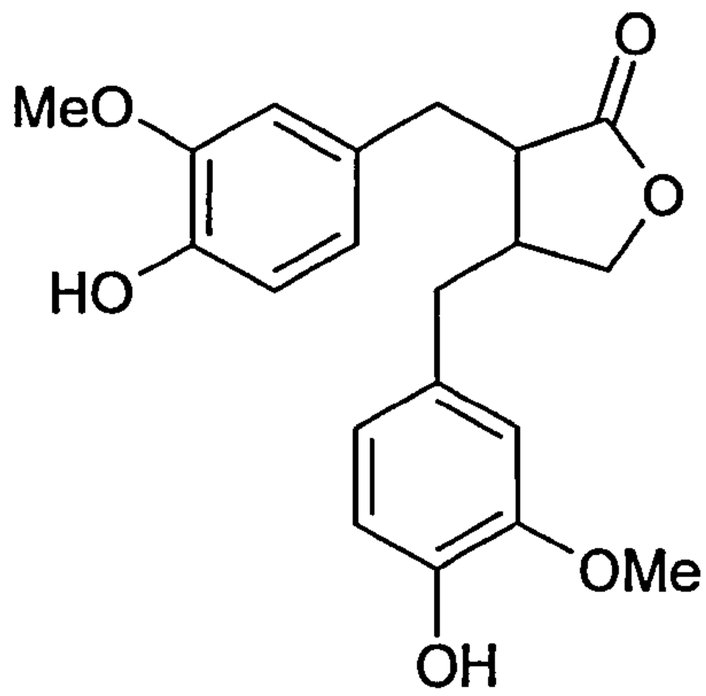
Sitosterol (**88**)



Genistein (**89**)



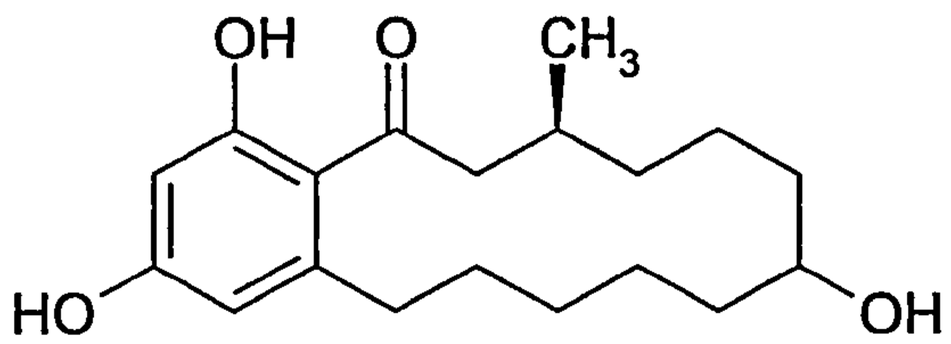
Coumestrol (**90**)



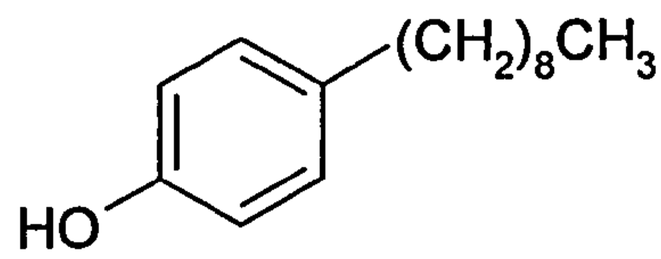
Matairesinal (**91**)

Other oestrogenic compounds, which have been identified from plant sources, include isoflavones (e.g. genistein (**89**)), coumestans (e.g. coumestrol (**90**)) and lignans (e.g. matairesinal (**91**)). Coumestrol (**90**) and genistein (**89**) showed oestrogenic activity in the recombinant yeast screen, but required concentrations 100- to 1000-fold greater than E2 (**14**) (Collins *et al.*, 1997). These compounds contain the phenolic hydroxyl substituent, considered important for binding to the hER. It has been reported that methylation of either hydroxyl substituent on coumestrol (**90**) reduces the oestrogenic activity (Dodge, 1998). Loss of activity may be due to the lack of a hydroxyl substituent to interact with ARG 394 in the LBD of the ER. Other structural modifications to coumestrol (**90**) that reduce oestrogenic activity include opening of the furan ring and increasing the number of hydroxyl substituents (Dodge, 1998). These occurrences may affect activity of coumestrol (**90**), by reducing the steric compatibility within the LBD.

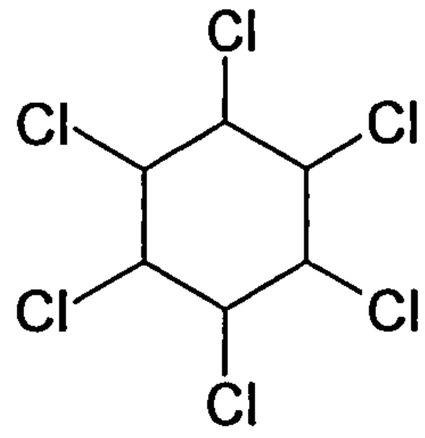
Several environmental oestrogens, including the macrolactones (e.g. zearalanol (**92**)) and the alkylphenols (e.g. nonyl phenol (**93**)), also contain a phenolic component in their structure, which has been considered a structural requirement for favourable binding to the hER. However, non-aromatic oestrogens have been identified, such as some halogenated carbocycle compounds (e.g. hexachlorocyclohexane (**94**)). However, hexachlorocyclohexane (**94**) has not been shown to bind to hER, although oestrogenic activity has been demonstrated *in vitro* (Dodge, 1998). Hexachlorocyclohexane (**94**) does contain a cyclic component, unlike the monoterpenes analysed for their oestrogenic potential in these investigations.



Zearalanol (**92**)



Nonyl phenol (**93**)



Hexachlorocyclohexane (**94**)

Another feature, which has been identified as important in determining the oestrogenic potency of phenolic additives, is molecular weight (Miller *et al.*, 2001). The oestrogenic potency of phenolic additives diminished as the molecular weight decreased from an optimum range of 200 - 230, with a molecular weight of 140 - 250 being proposed to favour oestrogenic activity (Miller *et al.*, 2001).

Citral (citral a (**70**) and citral b (**71**)), geraniol (**72**) and nerol (**75**) have molecular weights of 152, 154 and 154 respectively. These are not phenolic compounds, but their molecular weights do comply with the molecular weights (140 - 250) of phenolic additives suggested by Miller *et al.* (2001) to be important for oestrogenic activity. The relatively low molecular weights of these monoterpenes may also reflect their low potencies in the oestrogen bioassays (refer to 4.7, 4.8 and 4.9), as Miller *et al.* (2001) proposed that decreasing molecular weight may also reduce oestrogenic

potency. Eugenol (**84**) is a phenolic compound with a molecular weight of 164, and so complies with the appropriate molecular weight criteria for ER affinity, suggested by Miller *et al.* (2001), but the relatively low molecular weight may also reflect its relatively low affinity for ER in the bioassays (refer to 4.8 and 4.10). Interpretations of the possible link between molecular weight and ER affinity are not conclusive, as other parameters that may influence ER affinity including molecular structure and charge distribution, are not considered.

The proposed antagonist molecule eugenol (**84**) was investigated for potential binding properties in the LBD of the hER and compared to the binding of the antagonist molecule, RAL (**95**). The positioning of eugenol (**84**) in the LBD of ERα may favourably participate in hydrophobic interactions, as shown by the atomic van der Waals surfaces of the molecular systems, which show steric complementarity (Figure 4.39).

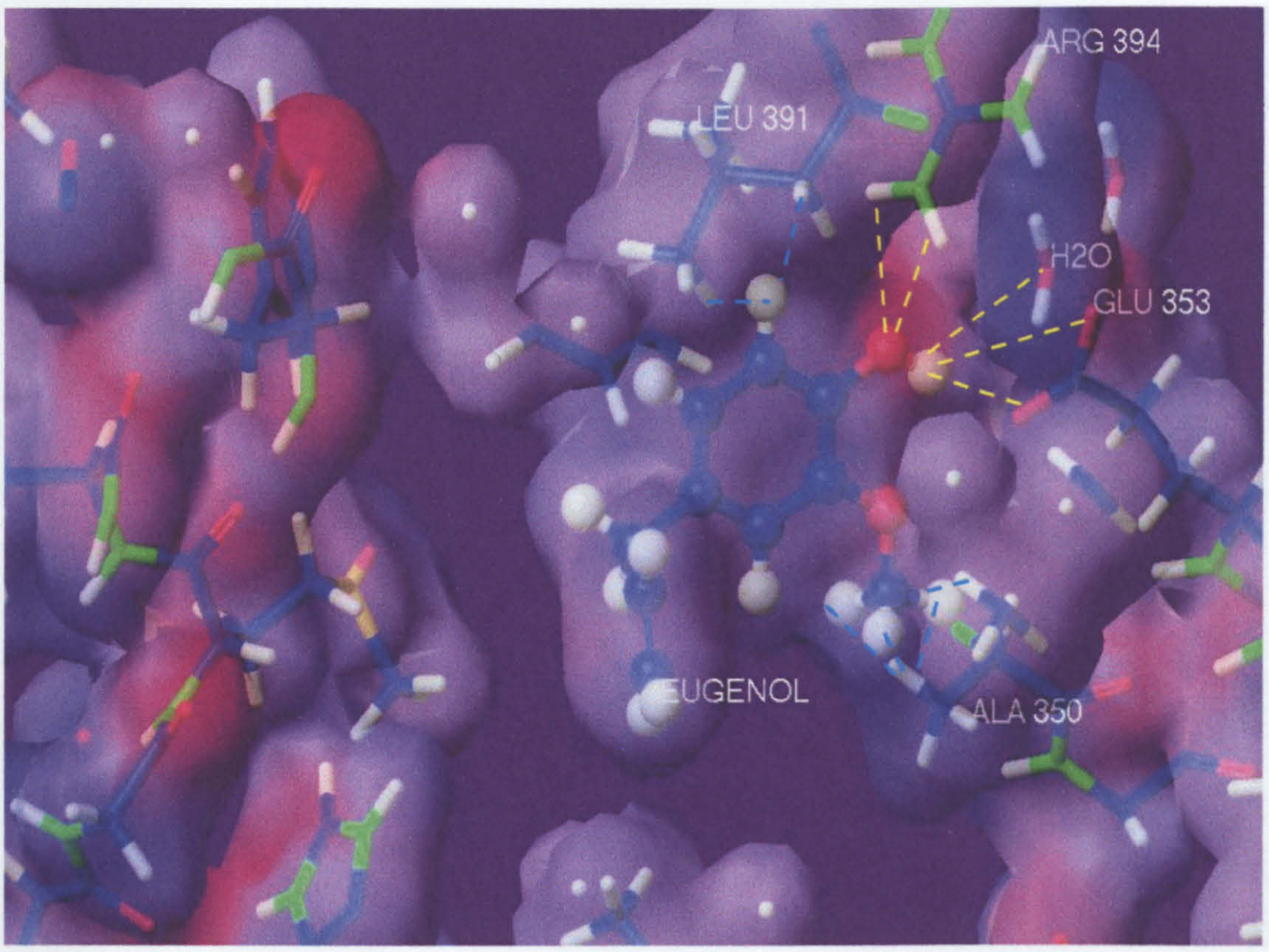


Figure 4.39. Potential interactions (- - - - -) of eugenol with the surrounding residues and potential displacement (- - - - -) of the surrounding residues by eugenol, in the ligand binding domain of the ERα.

The hypothetical binding mechanism of eugenol (**84**) may involve the interaction of the hydroxyl substituent with the guanidinium substituent of ARG 394, with the carboxylate substituent of GLU 353, and with a water molecule (shortest bond lengths: 2.05Å, 2.25Å and 3.15Å respectively) (Figure 4.39). However, for favourable binding to these amino acid residues of the LBD, eugenol (**84**) may displace other amino acid residues (leucine (LEU) 391 and alanine (ALA) 350), resulting in rotation and disruption of theses amino acid residues in the LBD. Helix displacement is regarded as a general feature of steroidal and non-steroidal hER antagonists (Brzozowski *et al*., 1997; Hendry *et al*., 1994; McDonnell, 1999; Wolf and Fuqua, 1995). For example, the imidazole ring of HIS 524 in the LBD rotates to accommodate the RAL (**95**) molecule, and maintain favourable hydrogen bonding between the hydroxyl substituent positioned at C11 of the RAL (**95**) molecule, and the imidazole ring of HIS 524 (Brzozowski *et al*., 1997 and Figure 4.40).

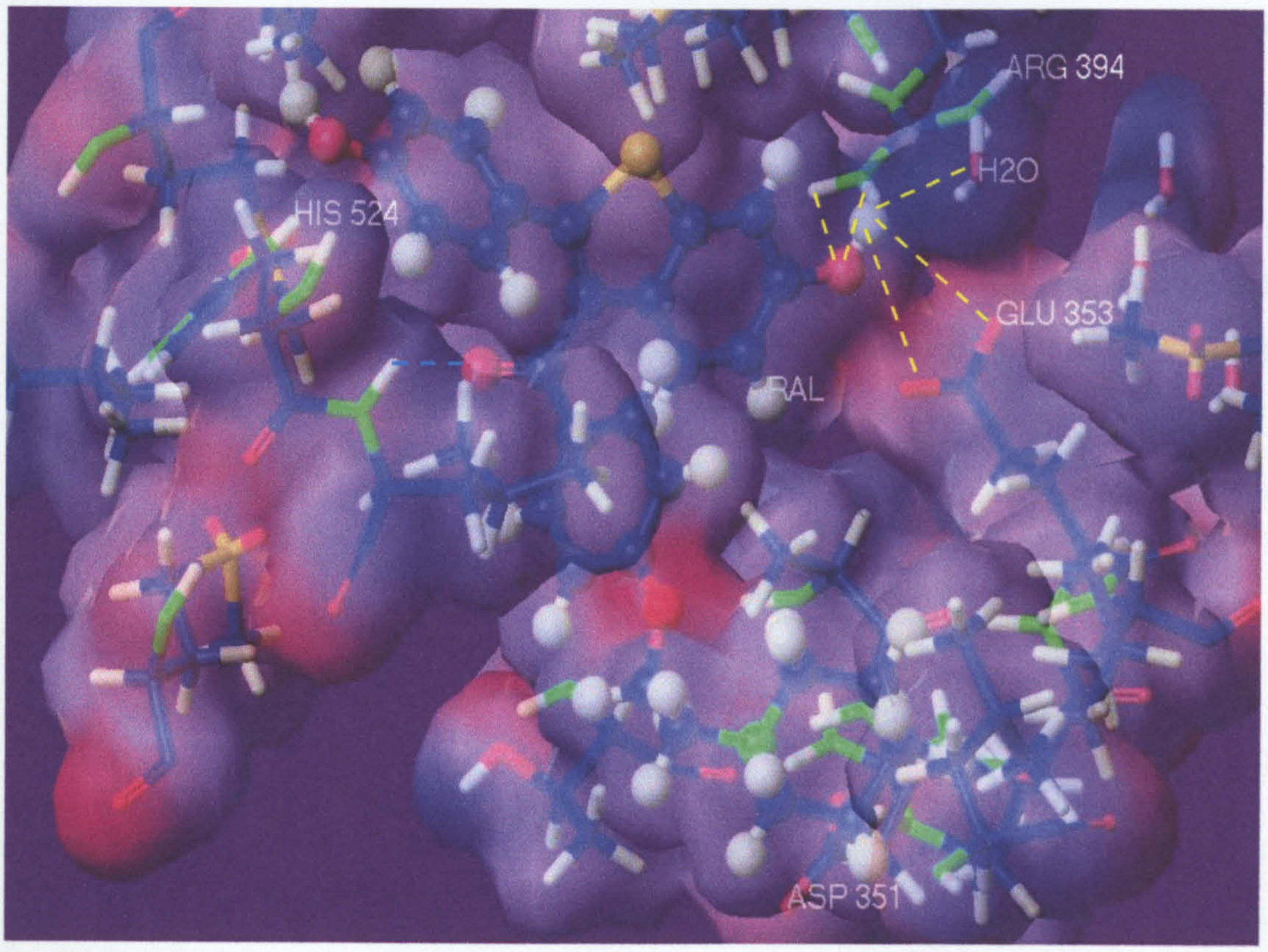
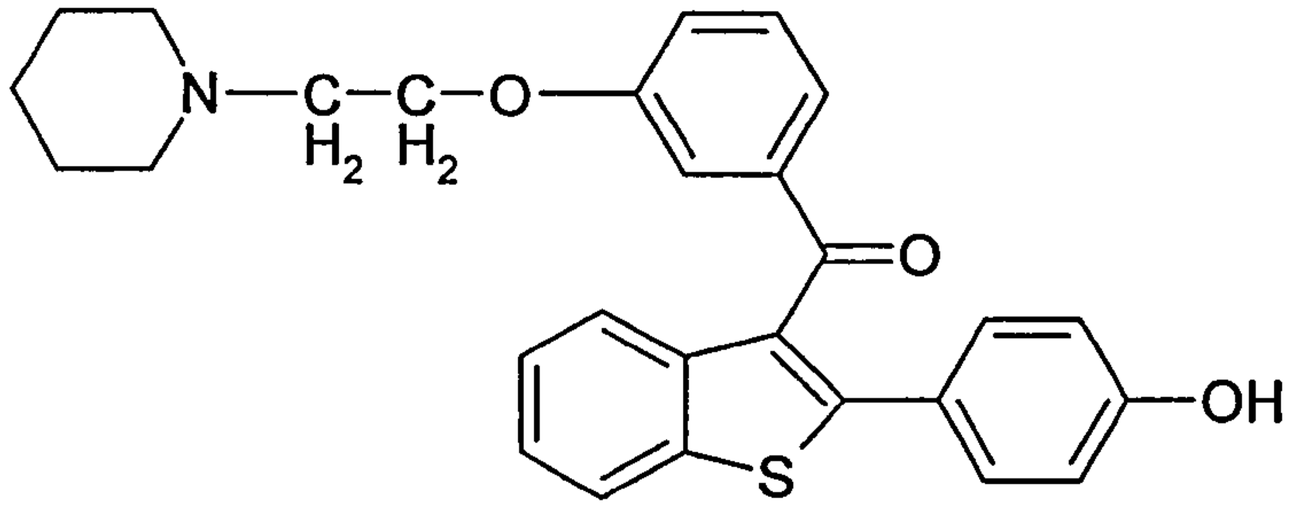


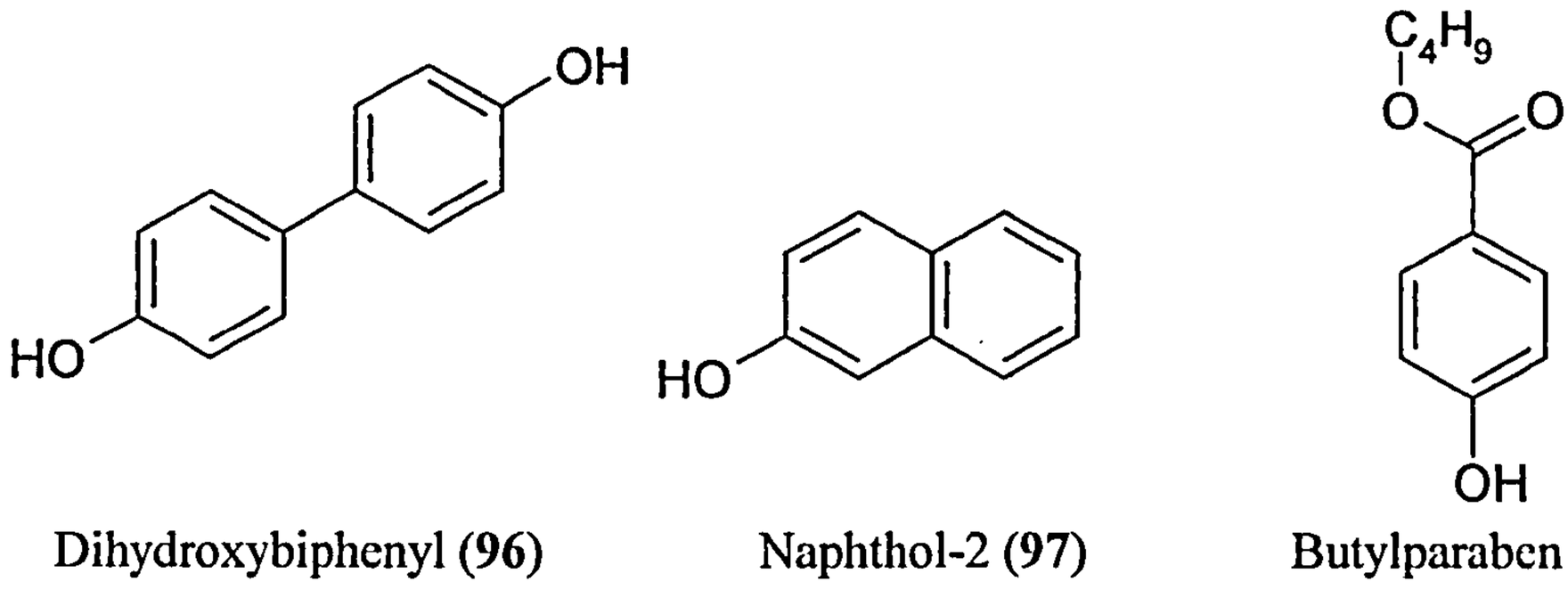
Figure 4.40. Potential interactions (- - - - -) of raloxifene (RAL) with the surrounding residues and potential displacement (- - - - - -) of the surrounding residues by RAL, in the ligand binding domain of the ERα.



Raloxifene (**95**)

Eugenol (**84**) is an alkyl phenolic compound. Several alkyl phenols (e.g. dihydroxybiphenyl (**96**) and naphthol-2 (**97**)) have been shown to displace [^{3}H]-17β-ostradiol binding to ERs (Blair *et al.*, 2000; Dodge, 1998; Mueller and Kim, 1978; Routledge and Sumpter, 1997). Some of the alkyl phenols investigated were agonistic (Miller *et al.*, 2001), but potential antagonistic effects of the alkyl phenols that did not show oestrogenic activity require further analysis. Some parabens (e.g. butylparaben (**98**)), chemically related to the alkyl phenols, have also shown affinity for ER, and showed weak oestrogenic activity in the recombinant yeast screen (Blair *et al.*, 2000; Routledge *et al.*, 1998).

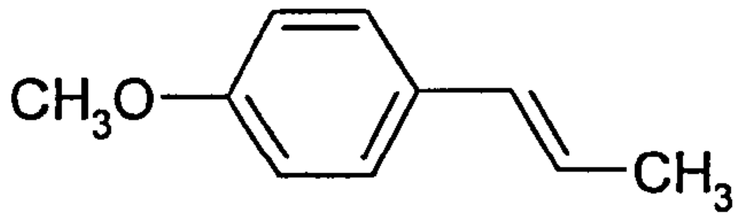
It is therefore not surprising that the alkyl phenol, eugenol (**84**) also displaced [^{3}H]-17β-ostradiol binding to ERα and ERβ in the *in vitro* receptor binding studies and showed antagonistic activity in the yeast screen (refer to 4.8 and 4.10); effects which may be explained by the proposed ER binding mechanism (Figure 4.39).



Dihydroxybiphenyl (**96**) Naphthol-2 (**97**) Butylparaben (**98**)

These studies using molecular graphics techniques appear to support the biological findings reported, regarding the oestrogenic and anti-oestrogenic properties of the monoterpene and phenylpropanoid compounds.

Another phenylpropanoid compound, anethole (**99**), has been suggested to be responsible for reported oestrogenic activity of fennel (*Foeniculum vulgare*) and anise (*Pimpinella anisum* L.) oils (Albert-Puleo, 1980; Zondek and Bergmann, 1938). However, the ligand binding effects of this compound remain to be established.



Anethole (**99**)

Compounds reported to possess oestrogenic and anti-oestrogenic effects may differ in their structure and their potential binding properties, from the typical cyclic molecules, such as the classical steroidal compounds. The investigation of potential ligands for the hER is further complicated by the presence of two ERs: ERα and ERβ (and possibly more ER subtypes, yet to be identified). The proposed interactions of the monoterpenes citral a (**70**), citral b (**71**), geraniol (**72**) and nerol (**75**), and the phenylpropanoid eugenol (**84**) for the LBD of ERα, contributes to the understanding of the complexity of ER binding and the subsequent oestrogenic and anti-oestrogenic responses.

4.12 Conclusion

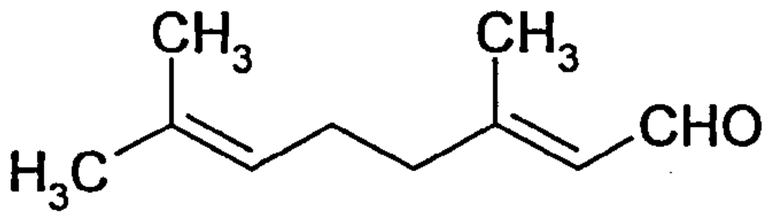
The oestrogenic activity of many plants, and compounds isolated from plants, is well documented (Dodge, 1998; Gehm *et al.*, 1997; Milligan *et al.*, 1998; Milligan *et al.*, 1999; Murkies *et al.*, 1998; Stevens *et al.*, 1998; Zava, 1998). The identification of oestrogenic activity in the root of *Polygala tenuifolia* has not previously been reported, but further studies are necessary to identify the compounds responsible for the apparent oestrogenic activity. Assessment of the oestrogenic activity of compounds isolated from *Polygala tenuifolia in vivo* is important to characterise potential ER agonists, which may be exploited for management of disorders such as AD. Beresford *et al.* (2000) emphasised the necessity of using a suite of assays to minimise the chances of wrongly labelling chemicals as endocrine disrupters. This

problem is well illustrated by the results of the present study in which oestrogenic activity was observed for the monoterpenes in some assays, but not others.

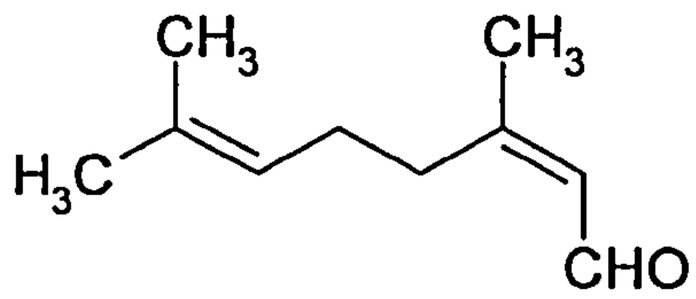
The binding affinity of the monoterpenes citral (geranial (**70**) and neral (**71**)), geraniol (**72**) and nerol (**75**) for the isolated ERα and ERβ has not previously been reported. The yeast bioassay provided evidence that affinity for the hER may be due to an oestrogenic effect. The results from the yeast screen and the receptor binding studies (refer to 4.7 and 4.8) support previous findings, which suggest citral may have oestrogenic activity (Abramovici *et al.*, 1987; Geldof *et al.*, 1992; Massas *et al.*, 1991; Servadio *et al.*, 1986), which raises questions concerning the physiological significance of exposure to these compounds.

Monoterpenes from the essential oils of plants have been reported to have a wide variety of biological properties. Citral and geraniol (**72**) induce malformation in chick embryos, affecting reproduction (Abramovici, 1972). In rats, topical application of citral has also been reported to induce infertility due to ovarian atrophy and oocyte loss (Toaff *et al.*, 1979), which may affect endocrine responses. Citral has been found to induce sebaceous gland hyperplasia in rats, which was proposed to have occurred by citral stimulating testosterone production (Sandbank *et al.*, 1988). Citral has also been proposed as a retinoic acid synthesis inhibitor, perhaps by inhibiting enzymes responsible for retinoic acid formation (Chen *et al.*, 1995; Connor and Smit, 1987; Hayden *et al.*, 2001; Kikonyogo *et al.*, 1999; Tanaka *et al.*, 1996).

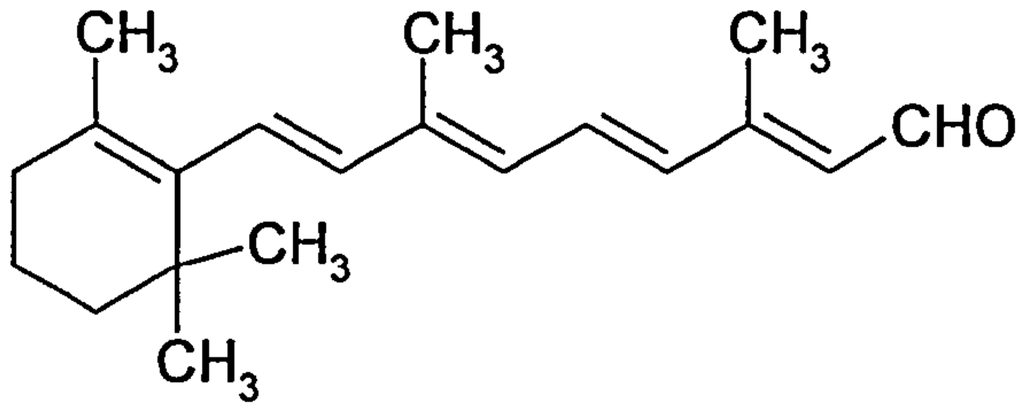
However, in view of the results from the present study, which suggest that citral may interact with the ER (refer to 4.7 and 4.8), the possibility is raised that citral may also interact with the retinoic acid receptor. The ER is a member of the steroid receptor family of transcription factors, which also includes receptors for vitamin D, thyroid hormone and retinoic acid, which share common structural features. It is interesting to note that geranial (**70**) and neral (**71**) are structurally similar to retinaldehyde (**100**). The effects of citral on tissues responsive to sex hormones, such as the prostate (Abramovici *et al.*, 1987), ovaries (Toaff *et al.*, 1979) and sebaceous glands (Abramovici *et al.*, 1982), suggest citral may act on a molecular level as a hormone. These findings support the results from this investigation, which shows citral may have an oestrogenic action (refer to 4.7 and 4.8).



Geranial (Citral a) (**70**)



Neral (Citral b) (**71**)



Retinaldehyde (**100**)

The potential anti-oestrogenic effect of eugenol (**84**) could be exploited for the development of new anti-oestrogens for disorders including hormone-dependent carcinogenesis; eugenol (**84**) showed greater affinity for ERα than ERβ (refer to 4.8.2), and ERα is reported to predominate in the breast, uterus and ovary (Mason, 2001). Eugenol (**84**) may not be appropriate for disorders such as AD, in which oestrogen agonists may be required to minimise cognitive decline.

However, there may be potential for the monoterpenes to act as oestrogens, or to act as templates for the development of other oestrogenic compounds, which may be relevant in the management of disorders such as AD. It is important to consider the potential oestrogenic effects of these compounds before they are used therapeutically or otherwise, as there is potential for adverse effects on health. It has previously been reported that monoterpenes from the essential oils of plants have anticarcinogenic effects (Crowell, 1999), but the inclusion of potentially oestrogenic essential oil constituents in a vast range of products may actually contribute to the development of reproductive disorders and cancer.

The essential oil constituents citral, eugenol (**84**), geraniol (**72**), and nerol (**75**) are derived from a variety of sources including fruits, vegetables and herbs. Consequently, such compounds are widely available for consumption. Essential oil constituents are also frequently included in cosmetics and household products. Their use in deodorants available on the European market has been analysed, and eugenol (**84**) and geraniol (**72**) were found to be present most frequently (57% and 76% respectively) in the products investigated (Rastogi *et al.*, 1998). Use of essential oils

in detergent products, and raised awareness of alternative therapies such as aromatherapy and herbalism have also increased exposure to many essential oils. Commercially available insect repellents have also increased human exposure to monoterpenes. For example, citronella oil (obtained from *Cymbopogon nardus*) is widely used as an insect repellent. Citronella oil contains geraniol (**72**) as a major constituent, reported to compose 20% - 41% of the total oil (Lawrence, 1988). Citriodol, an extract of lemon eucalyptus oil, is also frequently included in insect repellent products (Goodyer, 2000). It is therefore of importance that the effects of essential oil components on the environment and to health are assessed.

Like essential oil constituents, parabens are frequently included in a vast range of toiletries. Paraben preservatives have been shown to be oestrogenic agents *in vitro* and *in vivo* (Blair *et al.*, 2000; Routledge *et al.*, 1998). Emasculating effects in males and an increase in male reproductive disorders have been attributed to their inclusion in products to which humans are repeatedly exposed. Geraniol (**72**) has also been implied to cause oestrogenic activity *in vivo*, as gynecomastia has been reported in man following its use (Abramovici and Sandbank, 1988).

Therefore, although the experimental results show that the oestrogenic effects of citral, geraniol (**72**) and nerol (**75**) are far less potent than the endogenous hormone E2, repeated exposure may result in cumulative oestrogenic effects, or perhaps synergistic effects with other xenoestrogens *in vivo*.

CHAPTER 5

Investigations for Anti-Inflammatory Activity of Plant Extracts and Essential Oils

Arachidonic acid (AA) is metabolised by 5-lipoxygenase (5-LOX) to yield the leukotriene inflammatory mediators (e.g. LTB_4, LTC_4, LTD_4, LTE_4), and by cyclo-oxygenase (COX) to yield the prostaglandin (e.g. PGD_2, PGE_2, PGF_2) and thromboxane (e.g. TXA_2, TXB_2) inflammatory mediators (Figure 5.1). LTB_4 enhances lysosomal enzyme activity and induces chemotaxis; TXB_2 is involved in platelet aggregation.

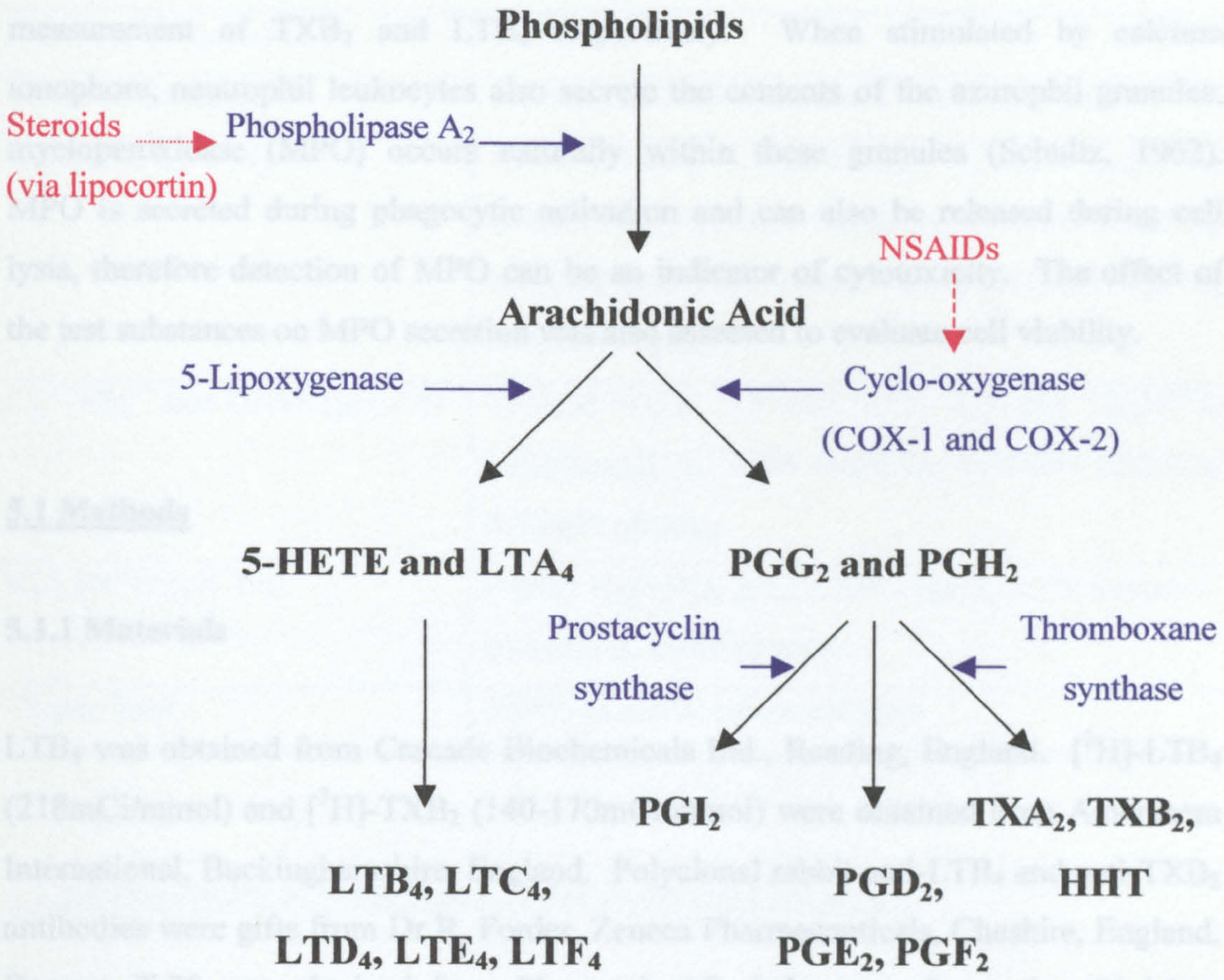


Figure 5.1. Sites of action of anti-inflammatory drugs (non-steroidal anti-inflammatory drugs (NSAIDs) and steroids) on arachidonic acid metabolism.

Consequently, inhibition of enzymes in the arachidonate cascade is an important therapeutic target to manage inflammatory disorders and, in view of current theories linking AD with inflammatory mechanisms (refer to Chapter 1, 1.2.3), may also be used therapeutically in this condition.

Crude aqueous and ethanolic plant extracts, essential oils and essential oil constituents, were assessed for their effects on leukocyte eicosanoid formation by investigating for activity against enzymes (COX and 5-LOX) of the arachidonate cascade.

A radioimmunosassay (RIA) was conducted using calcium ionophore stimulated rat peritoneal leukocytes, as described by Moroney *et al.* (1988). The rat peritoneal leukocytes employed for these investigations express pathways for both COX and 5-LOX, and inhibition of COX and 5-LOX by the test substances was assessed by measurement of TXB_2 and LTB_4 respectively. When stimulated by calcium ionophore, neutrophil leukocytes also secrete the contents of the azurophil granules; myeloperoxidase (MPO) occurs naturally within these granules (Schultz, 1962). MPO is secreted during phagocytic activation and can also be released during cell lysis, therefore detection of MPO can be an indicator of cytotoxicity. The effect of the test substances on MPO secretion was also assessed to evaluate cell viability.

5.1 Methods

5.1.1 Materials

LTB_4 was obtained from Cascade Biochemicals Ltd., Reading, England. [^{3}H]-LTB_4 (218mCi/mmol) and [^{3}H]-TXB_2 (140-170mCi/mmol) were obtained from Amersham International, Buckinghamshire, England. Polyclonal rabbit anti-LTB_4 and anti-TXB_2 antibodies were gifts from Dr R. Forder, Zeneca Pharmaceuticals, Cheshire, England. Dextran T-70 was obtained from Pharmacia-AB, Laboratory Separation Division, Uppsala, Sweden. Purified charcoal was obtained from Norit GSX. Hank's balanced salt solution (HBSS) was purchased from Gibco Ltd., Middlesex, England. Scintillation fluid (Liquiscint®) was obtained from National Diagnostics, Aylesbury, Buckinghamshire, England. Solvents were obtained from BDH Supplies, Poole,

England. All other chemicals were purchased from Sigma Chemical Company, Poole, Dorset, England.

Plant material, essential oils and essential oil constituents were obtained as described previously (Chapter 2, 2.1.1.1); *Melissa officinalis* essential oil (organic) was obtained from Fragrant Earth, Taunton, England.

5.1.2 Activity of Plant Extracts, Essential Oils and Essential Oil Constituents Against Leukocyte Eicosanoid Formation

5.1.2.1 Preparation of Assay Solutions

Table 5.1. Composition of assay solutions used for the anti-inflammatory assay.

Solution/Suspension	**Composition**
Charcoal suspension	1% charcoal, 0.5% dextran T-70 in RIA buffer
Complete HBSS (pH 7.4)	137mM NaCl, 5.37mM KCl, 0.34mM Na_2HPO_4 (anhydrous), 4.17mM $NaHCO_3$, 0.44mM KH_2PO_4, 5.55mM glucose, 1.26mM $CaCl_2$, 0.41mM $MgSO_4$, 0.49mM $MgCl_2$
Ca^{2+}/Mg^{2+} free HBSS (pH 7.4)	137mM NaCl, 5.37mM KCl, 0.34mM Na_2HPO_4 (anhydrous), 4.17mM $NaHCO_3$, 0.44mM KH_2PO_4, 5.55mM glucose
RIA buffer (pH 7.4)	0.04M $NaH_2PO_4.2H_2O$, 0.9% NaCl, 0.1% bovine-γ-globulin in distilled water
Trypan blue	0.05% in sterile saline solution

5.1.2.2 Preparation of Suspensions of Rat Peritoneal Leukocytes

A suspension of leukocytes containing approximately 85% polymorphonuclear leukocytes (PMNs) and 15% mononuclear cells was obtained as follows:

10ml 6% oyster glycogen in saline was injected (i.p.) into male Wistar rats (200g - 300g) to induce peritonitis. After 16hr - 20hr, rats were asphyxiated in a CO_2

chamber and 50ml HBSS (Ca^{2+}/Mg^{2+} free) (4°C) was injected into the peritoneal cavity. Following approximately 90s of vigorous massage, the peritoneal washing was removed and centrifuged at 4°C for 10min at 1500rpm. After centrifugation, the supernatant was discarded and the contaminating erythrocytes in the pellet were lysed if necessary by resuspension in 1ml 0.2% NaCl for a few moments, before adding 9ml saline, followed by further centrifugation at 4°C for 10min at 1500rpm. The pellet was resuspended in 10ml HBSS, and the cell suspensions were combined. 100µl cell suspension was diluted with 900µl of HBSS; 100µl of this suspension was further diluted with trypan blue solution (1:1). Cells were counted using a haemocytometer and the average number of cells per area (4 x 4) was recorded. The required cell density of 2.5×10^6 cells/ml was obtained as follows:

$$\text{final volume of cell suspension} = \frac{\text{average cell number} \times 20^* \times 10^4{}^{**} \times \text{volume of cell suspension}}{2.5 \times 10^6}$$

* dilution factor

** area in haemocytometer

To achieve the required cell density of 2.5×10^6 cells/ml, the cell suspension was made up to the final volume calculated, using complete HBSS. Cell viability based on trypan blue exclusion was > 95%.

5.1.2.3 Stimulation of the Release of Eicosanoids (LTB_4 and TXB_2) and Their Radioimmunoassay

Triplicate 0.5ml aliquots of the leukocyte suspension were preincubated at 37°C for 10min with 5µl of the test substance diluted in MeOH. Test substances and controls (Table 5.2) were added prior to addition of cells, except for volatile essential oils and compounds and the MeOH control, which were added after addition of cells. All tubes containing volatile substances were stoppered to prevent loss of the volatile constituents during incubation. Further controls were set up to control for the effect of the test substance (at the highest concentration) in the RIA (C11).

Table 5.2. Controls used for radioimmunoassay.

Control	**Compound**	**Volume added**
C1	cells alone (no compounds added)	-
C2	MeOH	5µl
C3	MeOH (added after cells)	5µl
C4	DMSO	5µl
C5	distilled H_2O	5µl
C6	CH_3COCH_3	5µl
C7	20µM indomethacin (COX inhibitor)	5µl
C8	20µM ZM 230487 (5-LOX inhibitor)	5µl
C9	20µM ZM 211965 (5-LOX inhibitor)	5µl
C10	triton X-100 (20%)	2µl
C11	Test substance added after incubation	5µl

After preincubation, 1µl calcium ionophore A23187 was added in DMSO to give a final concentration of 1µM (except to C1) to generate eicosanoid release. Following further incubation at 37°C for 10min, the cells were centrifuged at 4°C for 10min at 1500rpm, and the cell-free supernatants were decanted and frozen at -20°C. Supernatants were retained for analysis by RIA and for assessment of myeloperoxidase activity. RIAs were conducted using the supernatant as follows:

Aliquots (2µl for LTB_4, 15µl for TXB_2) of the thawed supernatant samples were subjected to RIA by adjusting the volume to 100µl volume with RIA buffer. A series of standard solutions were also subjected to RIA; unlabelled LTB_4 and TXB_2 standard solutions were assayed (3.125pg/ml - 5000pg/ml). 200µl polyclonal rabbit anti-eicosanoid serum (diluted 1:1500 for anti-TXB_2 and 1:2000 for anti-LTB_4) was added to each sample. Tracer was diluted prior to use with RIA buffer to give 10 nCi [^{3}H]-TXB_2 or 4 nCi [^{3}H]-LTB_4 per 100µl. 100µl of tracer was then added to samples and

standards, and also to a zero standard, which contained no eicosanoid. The solutions were vortex mixed prior to incubation at 4°C for 18hr. After incubation, 200µl dextran coated charcoal was added to samples to adsorb free eicosanoid. Samples were vortex mixed and incubated at 4°C for 15min. To separate bound eicosanoid from free eicosanoid, samples were centrifuged at 4°C for 30min. The supernatants were decanted into scintillation vials and 5ml of Liquiscint® scintillation fluid was added. Quantitation of [^{3}H]-TXB_2 or [^{3}H]-LTB_4 was conducted using a Canberra Packard 1900TR β-scintillation counter for 5min, followed by interpretation of the values using a standard curve prepared using various amounts of eicosanoid ranging from 3.125pg/ml to 5000pg/ml. Sensitivity of these assays was 5pg.

5.1.3 Data Analysis

Results were expressed as the percentage of the controls (n=24: C2, C3, C4, C5, C6) with cells stimulated by A23187 (the percentage of A23187 was calculated using mean ng/ml of LTB_4 or TXB_2). Results were also expressed as eicosanoid concentration (ng/ml). Data are presented as the mean (n=3 for test substances and positive controls (C7, C8, C9) ± SEM). Data were analysed using the student t test to determine the significance of the difference between the controls (C2, C3, C4, C5, C6) and the substance under investigation. Significance was regarded as $p<0.05$.

5.1.4 Myeloperoxidase Assay Method

5.1.4.1 Preparation of Assay Solutions

Table 5.3. Composition of assay solutions used for the myeloperoxidase assay.

Assay Solution	**Composition**
Phosphate buffer (pH 6) / hexadecyl-trimethylammonium bromide (HTAB)	6.62mM Na_2HPO_4 (anhydrous), 0.06M KH_2PO_4 containing 0.5% HTAB
o-Dianisidine solution	*o*-dianisidine in distilled water (0.68mg/ml)
Hydrogen peroxide solution	H_2O_2 in distilled water (0.003%)

5.1.4.2 Myeloperoxidase Assay Method

Assessment of MPO activity was conducted as described by Bradley *et al.* (1982) and the Worthington Enzyme Manual (1972). The following reagents were added in the order stated to wells of a 96-well microtiter plate, in triplicate: 50µl leukocyte supernatant, 50µl phosphate buffer (pH 6, containing HTAB to solubilise MPO) and 50µl *o*-dianisidine solution. The reaction was initiated by addition of 50µl freshly prepared H_2O_2 solution to each well, and the optical density at 450nm was measured immediately, and at 1min, 3min, 6min, 15min and at 30min after the reaction was started. The optical density was measured using an Anthos HT II plate reader.

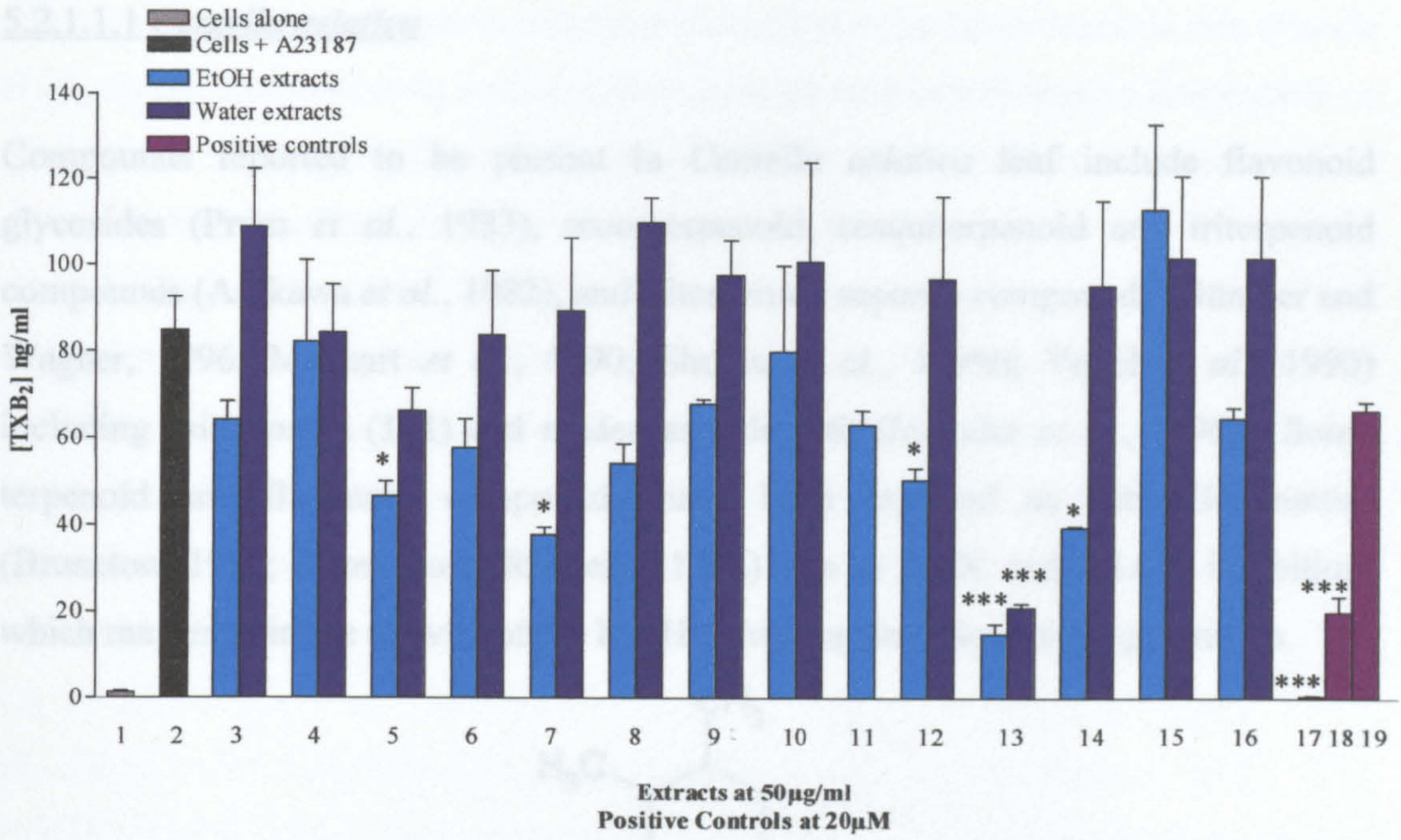
5.2 Results and Discussion

5.2.1 Activity of Plant Extracts Against Leukocyte Eicosanoid Formation

5.2.1.1 Activity of Plant Extracts Against Leukocyte TXB_2 Formation

Activation by ionophore A23187 of the peritoneal leukocytes caused a 65-fold increase in TXB_2 generation, an effect completely inhibited by the COX inhibitor indomethacin at 20µM (Figure 5.2). Aqueous and EtOH plant extracts were investigated at a concentration of 50µg/ml to determine their activity against leukocyte TXB_2 formation.

The results show that the EtOH extracts of *Centella asiatica* leaf, *Convallaria majalis* leaf, *Rosmarinus officinalis* leaf (dried) and *Withania somnifera* root were significantly active against TXB_2 formation ($p<0.05$) as were the EtOH and aqueous extracts of *Salvia miltiorrhiza* root ($p<0.001$); these extracts reduced TXB_2 generation by 45%, 56%, 40%, 53%, 82% and 75% respectively (Figure 5.2).



Key

1: Cells alone (C1)

2: Cells + A23187 (C2 - C6)

3: *Alisma orientalis* root

4: *Apocynum lancifolium* leaf

5: *Centella asiatica* leaf

6: *Codonopsis pilulosa* root

7: *Convallaria majalis* leaf

8: *Melissa officinalis* leaf

9: *Polygala tenuifolia* root

10: Adulterated *Polygonum multiflorum* root (*Gentiana* spp.)

11: *Rosmarinus officinalis* fresh leaf

12: *Rosmarinus officinalis* dried leaf

13: *Salvia miltiorrhiza* root

14: *Withania somnifera* root

15: *Ziziphus jujuba* seed

16: *Ziziphus jujuba* var *spinosa* seed

17: Indomethacin (C7)

18: ZM 230487 (C8)

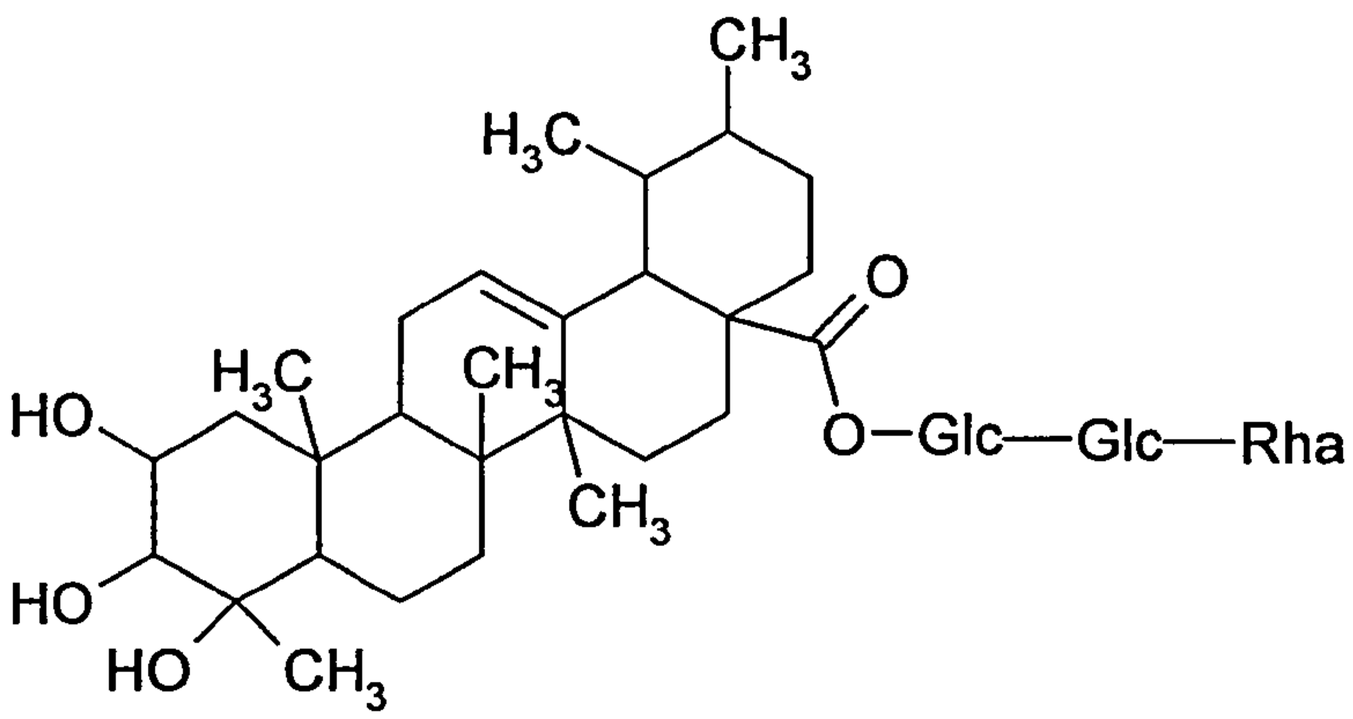
19: ZM 211965 (C9)

* $p<0.05$; ** $p<0.01$; *** $p<0.001$

Figure 5.2. Effect of aqueous and ethanolic plant extracts and three reference compounds on TXB_2 generation in rat peritoneal leukocytes (n=3, except cells alone: n=6 and A23187 controls: n=24).

5.2.1.1.1 *Centella asiatica*

Compounds reported to be present in *Centella asiatica* leaf include flavonoid glycosides (Prum *et al.*, 1983), monoterpenoid, sesquiterpenoid and triterpenoid compounds (Asakawa *et al.*, 1982), and triterpenoid saponin compounds (Günther and Wagner, 1996; Maquart *et al.*, 1990; Shukla *et al.*, 1999b; Vogel *et al.*, 1990) including asiaticoside (**101**) and madecassoside (**26**) (Inamdar *et al.*, 1996). Some terpenoid and flavonoid compounds have been reported as anti-inflammatory (Bruneton, 1995; Kenner and Requena, 1996) due to COX and 5-LOX inhibition, which may explain the activity of the EtOH extract against eicosanoid generation.

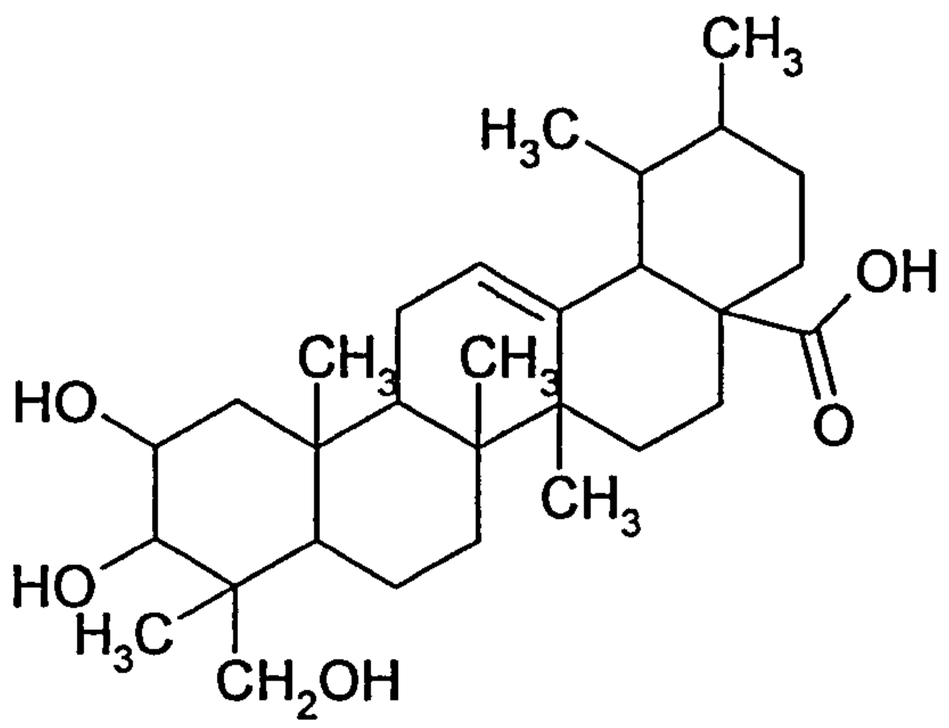


Asiaticoside (**101**)

The monoterpenes α-pinene (**53**) and β-pinene (**54**) are reported to be present in the essential oil from *C. asiatica* (Asakawa *et al.*, 1982), and may have been present in the EtOH extract. These monoterpenes are reported to be anti-inflammatory (Handa *et al.*, 1992).

Saponins are surface-active glycosides, so can potentially alter cell membrane structure and thereby cause haemolysis of erythrocytes (Rosenthal and Janzen, 1979). Lysis of leukocyte membranes may influence eicosanoid generation. However the relative selectivity of *C. asiatica* EtOH extract for the COX enzyme (Figures 5.2 and 5.3) suggests that the surfactant effect was not responsible for the apparent activity (the glycosides e.g. asiaticoside (**101**) may also have been at a higher concentration in the inactive aqueous extract). Some plants containing saponins are reported to be anti-inflammatory, perhaps because saponins have a similar molecular structure to steroidal compounds (e.g. barbatosides A and B from *Dianthus barbatus*) (Handa *et al.*, 1992; Kenner and Requena, 1996). The triterpene saponin glycyrrhizin from

Glycyrrhiza spp. is reported to potentiate the action of hydrocortisone in rats (Handa *et al.*, 1992), so it cannot be excluded that saponins may inhibit hepatic metabolism of corticosteroids *in vivo* and enhance steroidal activity via this mechanism, and not by a direct steroidal action on phospholipase A_2 activity. However, the topical administration of some saponins mimics the anti-inflammatory effects of corticosteroids. It has been reported that saponins are effective in the treatment of inflammatory skin conditions, such as atopic dermatitis and eczema (Jiménez *et al.*, 1987), perhaps due to steroidal activity. The triterpene asiatic acid (**102**) is reported to increase tensile strength of skin wounds, an effect that was attributed to steroidal structure of this compound (Weber, 1992).



Asiatic acid (**102**)

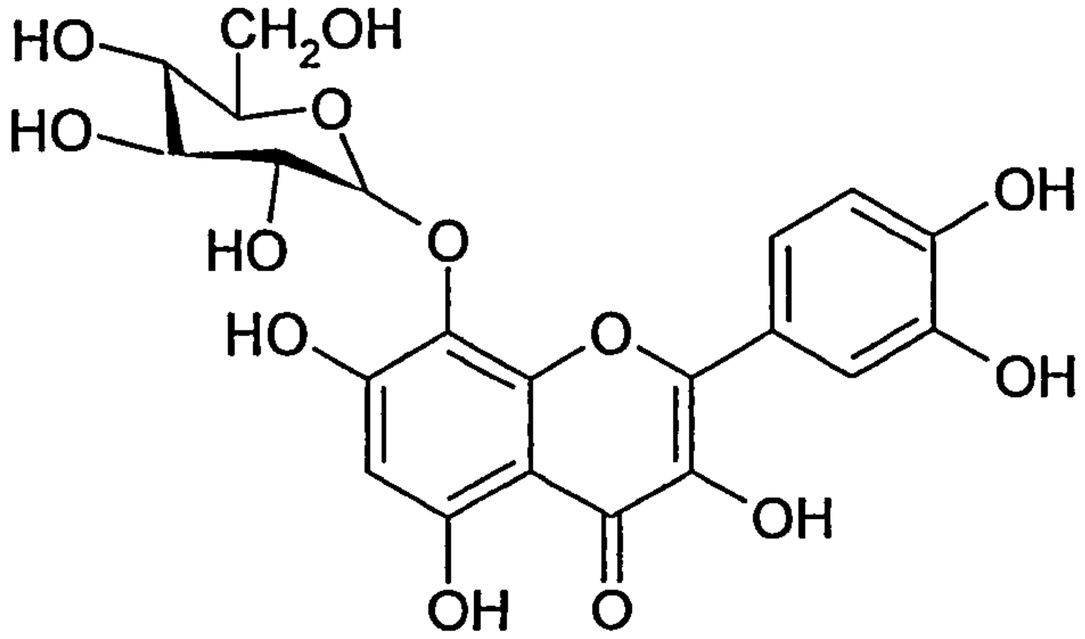
This biological activity of asiatic acid (**102**) may be evidence that steroidal compounds from *C. asiatica* are pharmacologically similar to anti-inflammatory steroidal drugs, and may influence *in vitro* eicosanoid generation, this effect being possibly due to activity against phospholipase A_2. *C. asiatica* is reportedly used in the Fiji Islands to treat inflammatory eye conditions (Brinkhaus *et al.*, 2000), which may be explained by the results of these investigations.

5.2.1.1.2 *Convallaria majalis*

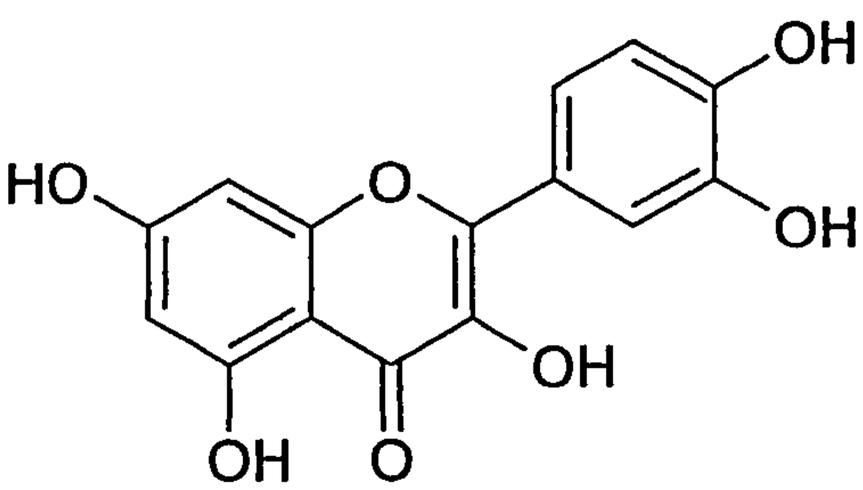
Convallaria majalis leaf has not previously been reported to have potential anti-inflammatory effects. Compounds identified in the leaf include cardiac glycosides and flavonoid glycosides (Trease and Evans, 1996). The aglycones of the cardiac glycosides are steroidal compounds (e.g. convallatoxin (**50**) yields the aglycone strophanthidin (**47**) and (-)-rhamnose upon hydrolysis). These steroidal aglycones

(which would be expected to be present in the active EtOH extract, but not the inactive aqueous extract) may be responsible for anti-inflammatory activity, perhaps by influencing phospholipase A_2 activity, like the anti-inflammatory corticosteroid drugs. However, the greater selectivity for the COX enzyme, over 5-LOX (Figures 5.2 and 5.3), suggests COX inhibiting compounds are present in the EtOH extract.

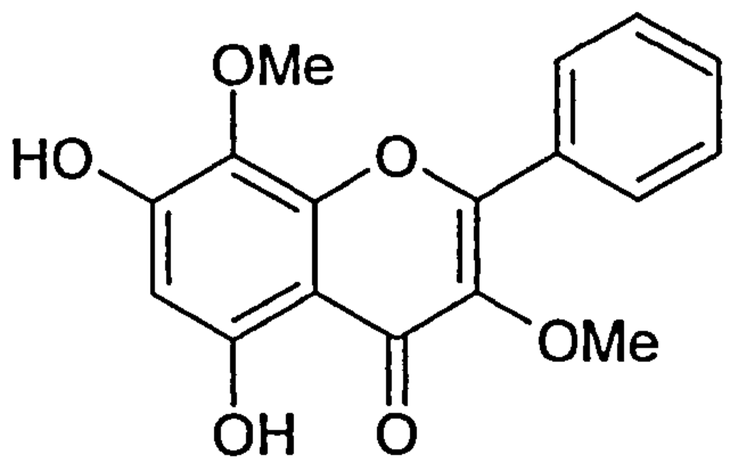
Many flavonoid compounds are reported to be anti-inflammatory, such as the flavonoid glycoside gossypin (**103**) from *Gossypium indicum*, and the flavonoid quercetin (**104**) from the Solanaceae (Harborne and Baxter, 1993). The lipophilic flavonol aglycone, gnaphalin (**105**), has been shown to inhibit LTB_4 and TXB_2 generation in rat peritoneal leukocytes (de la Puerta *et al.*, 1999). The structural features of flavonoid molecules required for COX inhibition have been reported to be the presence of a pyrocatechol group in at least one of the rings (A or B). For example, in flavonols hydroxylation at the 3 position is sufficient (Alcaraz and Ferrándiz, 1987). However, flavones without any such substituents can also inhibit COX (Mower *et al.*, 1984; Welton *et al.*, 1986).



Gossypin (**103**)



Quercetin (**104**)



Gnaphalin (**105**)

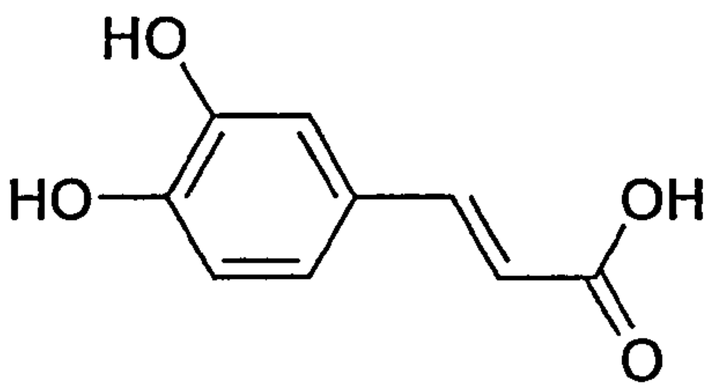
Therefore, it is possible that flavonoid compounds present in *C. majalis* leaf are responsible for COX inhibition, although activity due to other phytochemicals is possible. Other phenolic compounds, such as the coumarins, may also contribute to

anti-inflammatory activity (however, coumarins are yet to be isolated from *C. majalis* leaf). Some coumarins are also inhibitors of both COX and 5-LOX enzymes (Hoult and Payà, 1996).

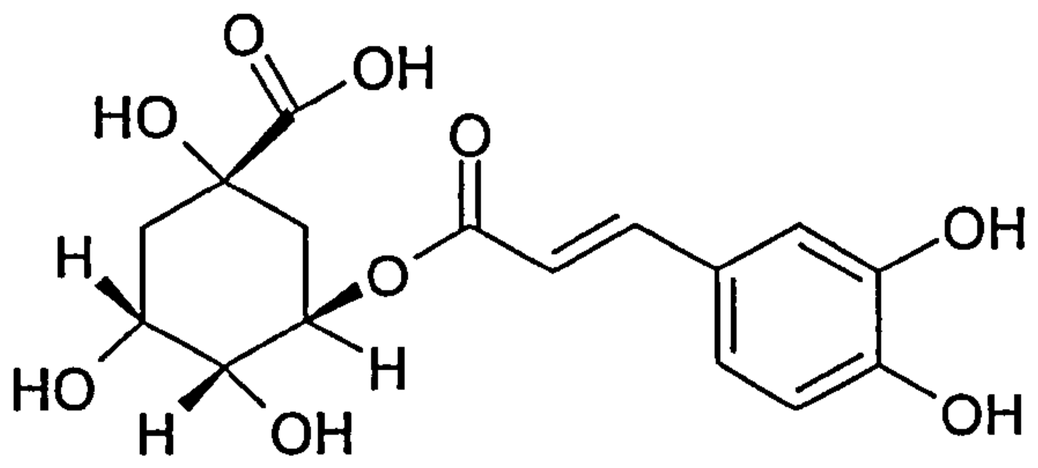
5.2.1.1.3 *Rosmarinus officinalis*

Rosmarinus officinalis leaf constituents identified include flavonoids (e.g. apigenin, luteolin (**114**)), phenolic compounds (e.g. caffeic acid (**106**) and chlorogenic acid (**107**)), diterpenoid (e.g. carnosol (**142**)) and triterpenoid compounds (e.g. oleanic acids and rofficerone), and a volatile oil composed mainly of monoterpenes (Bisset, 1994; Ganeva *et al.*, 1993; Newall *et al.*, 1996). The herb is recommended for rheumatoid arthritis in the German Commission E monographs (Bisset, 1994), which perhaps indicates that it contains anti-inflammatory compounds.

The EtOH extract of the fresh leaf of *R. officinalis*, and the aqueous extract of the dried leaf of *R. officinalis* were not significantly active against TXB_2 generation (Figure 5.2). This suggests that compounds responsible for activity were less polar than those compounds present in the aqueous extract (e.g. glycosidic compounds), and that the chemical composition of the herb may be affected by factors such as the storage conditions (e.g. light, humidity, temperature, duration of storage).



Caffeic acid (**106**)

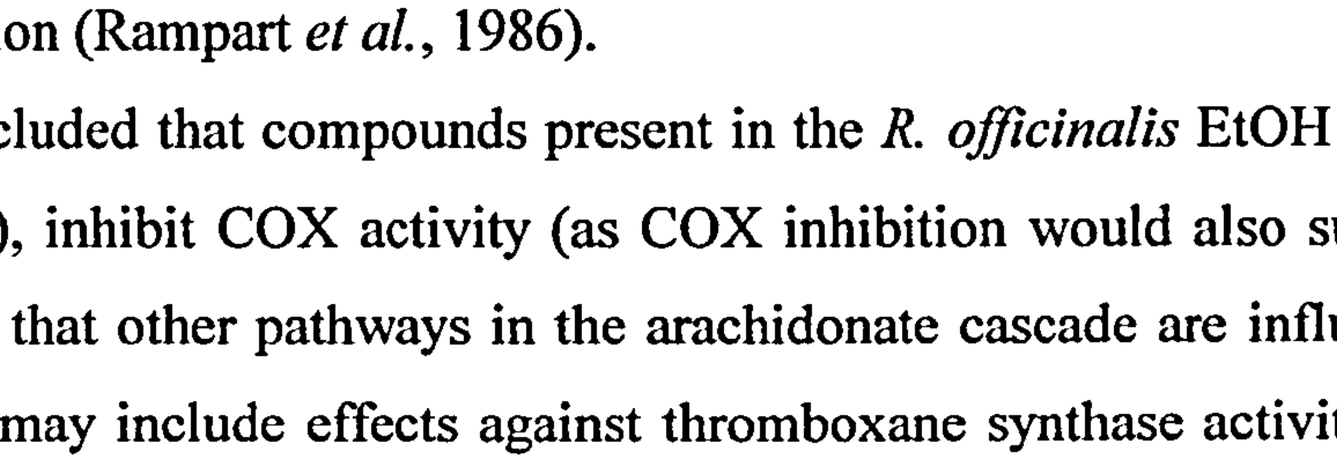


Chlorogenic acid (**107**)

Compounds present in the EtOH extract of the dried herb may therefore be COX inhibitors. However, previous studies have shown that rosmarinic acid (RA) (**108**), a compound found to be present in *R. officinalis* leaf, enhances PGE_2 formation in polymorphonuclear leucocytes (Kimura *et al.*, 1987). RA (**108**) and plant extracts containing RA (**108**) are scavengers of free radicals (Lamaison *et al.*, 1991). This activity may explain the prevention of the inactivation of COX by oxygen radicals. RA (**108**) has also been reported to influence the complement system, a system

involved in the inflammatory response. RA (**108**) was shown to inhibit cutaneous anaphylaxis in rats, impaired *in vivo* activation of macrophages in mice and reduced paw oedema induced by cobra venom factor (CVF) in rats; RA (**108**) did not inhibit oedema induced by *t*-butyl-hydroperoxide in the rat (Englberger *et al.*, 1988). This indicates selectivity for complement dependent processes, as CVF can selectively activate C3 and C5. Further experiments *in vitro* showed that RA (**108**) inhibited C3-convertase in the complement pathway (Englberger *et al.*, 1988), but it did not inhibit COX activity in studies investigating TXB_2 and 17-hydroxyheptadecatrienoic acid (HHT) formation (Rampart *et al.*, 1986).

It may be concluded that compounds present in the *R. officinalis* EtOH extract, other than RA (**108**), inhibit COX activity (as COX inhibition would also suppress PGE_2 formation), or that other pathways in the arachidonate cascade are influenced by the extract. This may include effects against thromboxane synthase activity. Inhibition of thromboxane synthase activity by RA (**108**) has not been established, however RA (**108**) does not inhibit prostaglandin synthase activity (Kuhnt *et al.*, 1995), an enzyme involved in PGI_2 synthesis.



Rosmarinic acid (**108**)

5.2.1.1.4 *Salvia miltiorrhiza*

Both the aqueous and EtOH extracts of *Salvia miltiorrhiza* root exhibited superior activity against TXB_2 generation ($p<0.001$), compared to the other extracts. Activation by ionophore A23187 of the peritoneal leukocytes caused a 63-fold increase in TXB_2 generation, which was reduced by 82% by the EtOH extract of *S. miltiorrhiza* root, and by 75% by the aqueous extract of *S. miltiorrhiza* root (Figure 5.2). *S. miltiorrhiza* root is used in TCM for rheumatoid arthritis and other inflammatory conditions (Duke and Ayensu, 1985). The scientific basis for these

indications may be explained to some extent by the activity against eicosanoid generation observed in these experiments. *S. miltiorrhiza* root extracts were also assessed for inhibitory activity against eicosanoid generation over the concentration range 25µg/ml - 200µg/ml (refer to 5.2.1.3).

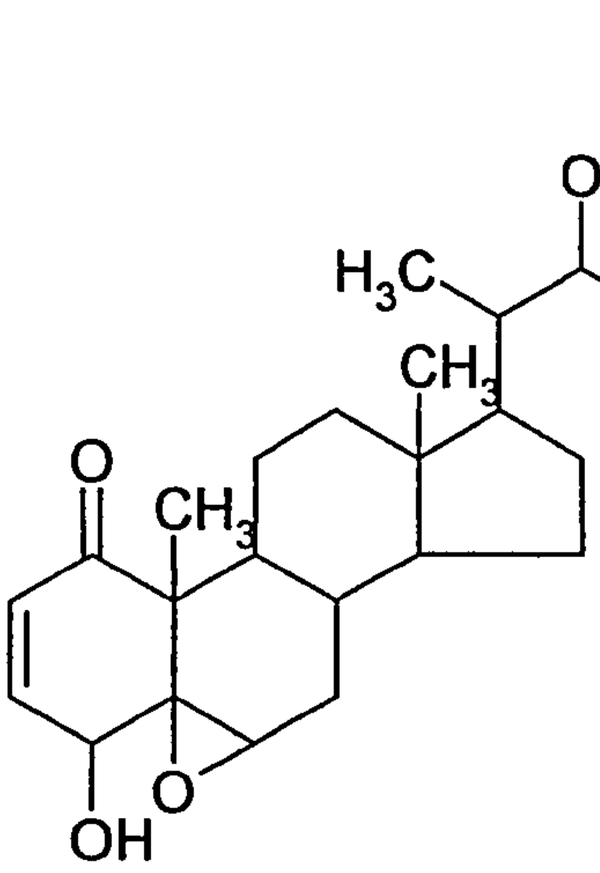
5.2.1.1.5 *Withania somnifera*

Withania somnifera root has been used in the Unani tradition for asthma (a pulmonary disease involving inflammation), bronchitis, arthritis and other inflammatory disorders (Asthana and Raina, 1988; Upton, 2000). *W. somnifera* root extracts have previously demonstrated immunomodulatory effects *in vivo*, including effects on immune inflammatory systems such as inhibition of ovalbumin induced paw oedema (Agarwal *et al.*, 1999). A traditional Ayurvedic prescription containing *W. somnifera* root, *Boswellia serrata* stem, *Curcuma longa* rhizomes and a zinc complex (Articulin-F) produced significant pain relief and decreased disability score in a randomised, double-blind, placebo-controlled, cross-over study, in patients with osteoarthritis (Kulkarni *et al.*, 1991). All herbal components of Articulin-F are reported to be either analgesic or anti-inflammatory. These herbs may act synergistically, or one herb (or compounds) may contribute more significantly to activity. To explain the observations in osteoarthritis patients, assessment of the herbs individually for potential activities relevant to disease management would be required.

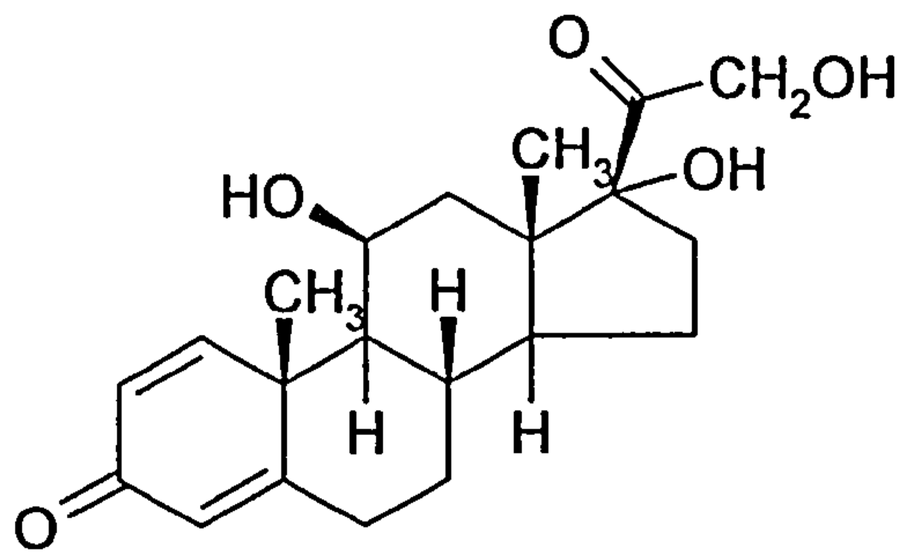
The leaves of *W. somnifera* suppress granulation tissue formation induced by cotton-pellet implantation in rats; this effect was attributed to steroidal compounds present in the plant, of which, withaferin A (**109**) is a major component (Al-Hindawi *et al.*, 1992). A compound, 3β-hydroxy-2, 3-dihydro-withanolide F, has been isolated from the fruits of another *Withania* spp. (*W. coagulans*); the anti-inflammatory activity (assessed in rats with formalin induced arthritis) of this compound was comparable to hydrocortisone and phenylbutazone (Budhiraja *et al.*, 1984). Related compounds, withanolides A - F are reported to occur in *W. somnifera* root (Upton, 2000) and may therefore have similar biological activities against inflammation.

These steroidal compounds (withanolides) may possess similar activity to corticosteroids, which are often prescribed for inflammatory conditions. This may be explained by the steroidal skeleton of the withanolides resembling the chemical structure of anti-inflammatory steroidal compounds, such as prednisolone (**110**).

Corticosteroids suppress eicosanoid generation by induction of the phospholipase A_2 inhibitors, lipocortins (Dale *et al.*, 1994).



Withaferin A (**109**)



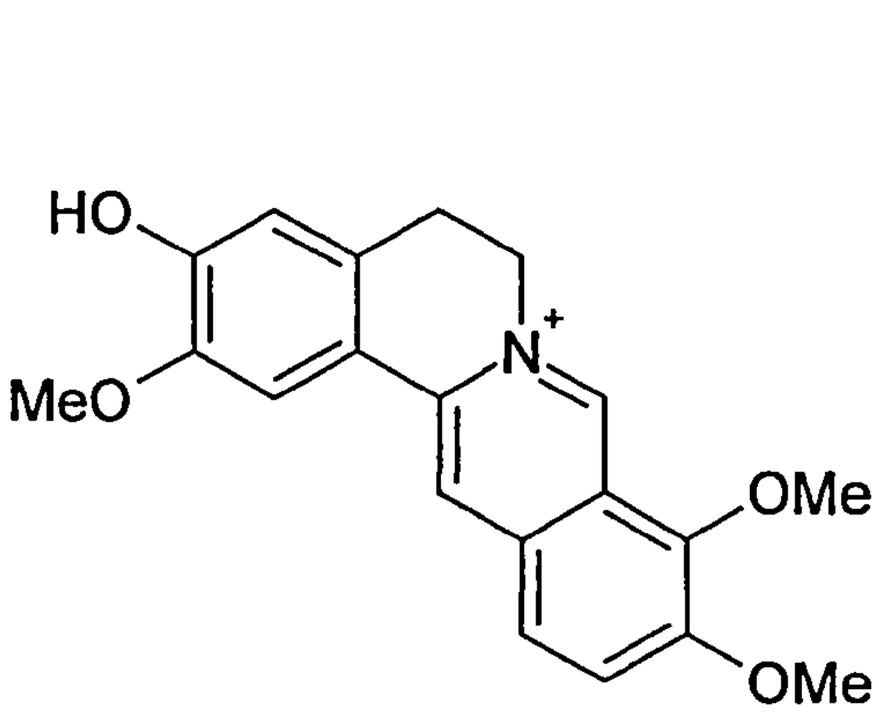
Prednisolone (**110**)

An alcohol extract from *W. somnifera* leaves showed comparable anti-inflammatory effects to phenylbutazone (a NSAID) and hydrocortisone against formalin-induced oedema and granulation tissue formation in rats (Sudhir *et al.*, 1986). Further investigations have shown *W. somnifera* root to be anti-inflammatory. The root was effective against arthritis in rats where a reduction in rat paw oedema and the prevention of bony degeneration was observed (Begum and Sadique, 1988). α2-Macroglobulin serum levels (an indicator of arthritis and inflammatory conditions) were reduced following treatment with *W. somnifera* root (Anbalagan and Sadique, 1981a; Anbalagan and Sadique, 1981b; Anbalagan and Sadique, 1985). The effect of the herb on acute phase reactants (APRs) (serum proteins which are elevated in inflammatory conditions), such as α2-macroglobulin, indicates that *W. somnifera* may also affect disease progression, and not just provide symptomatic relief, unlike the NSAIDs. The potential for the suppression of inflammatory processes could be of particular importance in delaying AD progression.

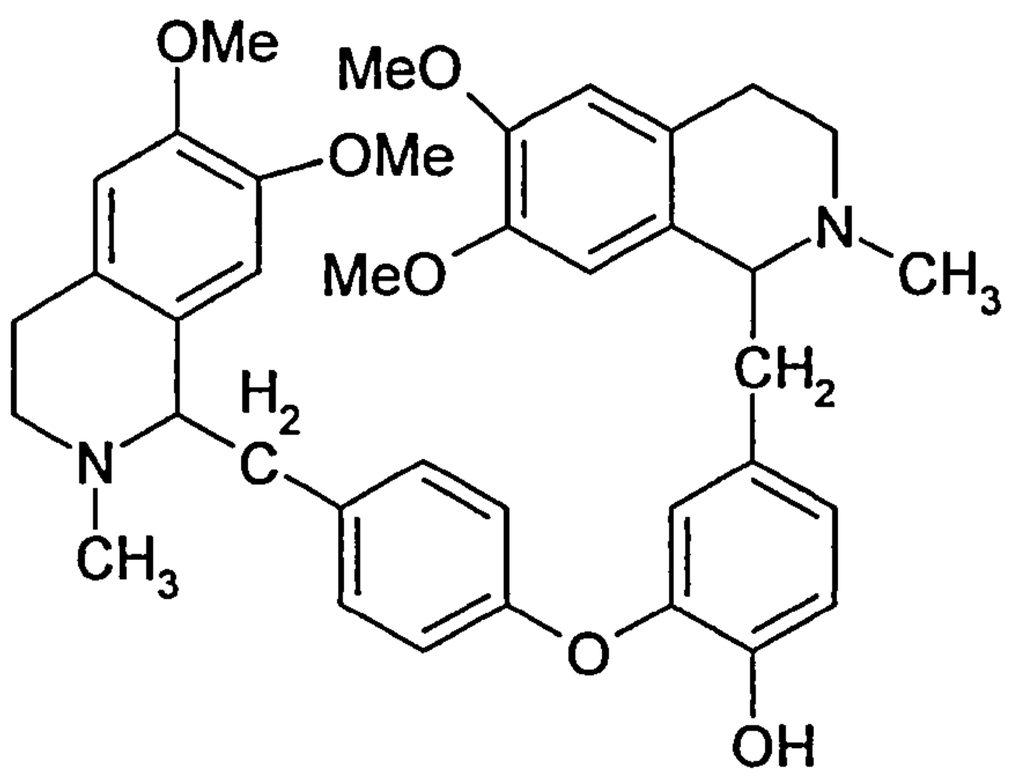
The observed effect of the EtOH extract of *W. somnifera* root against TXB_2 formation provides further evidence for the anti-inflammatory effects reported. Previous studies suggest the steroidal compounds present in the root and leaves of *W. somnifera* are responsible for anti-inflammatory activity. Treatment with NSAIDs is reported to have little or no effect on APRs, unlike the steroidal drugs (Anbalagan and Sadique, 1981a); this occurrence is further evidence for a steroidal anti-inflammatory activity

of *W. somnifera*. It could also be possible that compounds present can act similarly to the lipocortins and influence phospholipase A_2 activity directly. However, as the EtOH extract of *W. somnifera* root was more active against TXB_2 generation than LTB_4 generation (Figures 5.2 and 5.3), it is apparently more selective for COX (as opposed to 5-LOX). This suggests COX inhibitors are also present in the root (e.g. the anti-inflammatory flavonoid quercetin (**104**), which has been reported to be present in many plant families including the Solanaceae (Harborne and Baxter, 1993)).

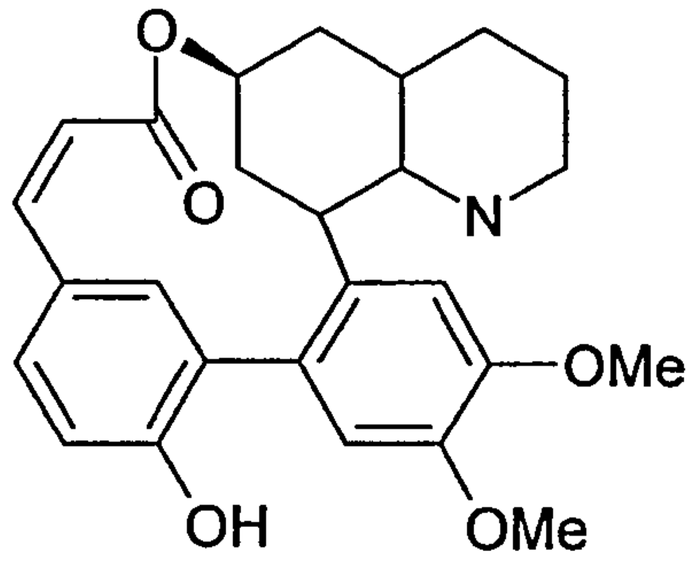
Numerous alkaloids have been identified in *W. somnifera* root (e.g. anaferine (**60**), ashwagandhine, cuscohygrine (**61**), somniferinine, withaninine and withasomine (Harborne and Baxter, 1993; Mills and Bone, 2000; Schwarting *et al.*, 1963; Upton, 2000)). Several alkaloids from various plant sources are reported to be anti-inflammatory, such as tomatine, a steroidal alkaloid glucoside from *Solanum lycopersicum*, which is also a member of the Solanaceae (Bingöl and Şener, 1995; Handa *et al.*, 1992). It is not only the steroidal alkaloids that have anti-inflammatory activity, which suggests that alkaloids may have other modes of action, besides effects on phospholipase A_2 activity. Jatrorrhizine (**111**) from *Plagiorhegma dubium*, dauricine (**112**) from *Menispermum dauricum* and tetrandrine from *Stephania tetrandra* are isoquinolone alkaloids, reported to be anti-inflammatory. Cryogenine (**113**) isolated from *Heimia salicifolia* is an inhibitor of prostaglandin synthetase (Bingöl and Şener, 1995; Handa *et al.*, 1992; Harborne and Baxter, 1993). The effects of *W. somnifera* against eicosanoid generation may have in part, been due to the alkaloids present. It is therefore possible that this herb may have more than one mode of action against inflammation.



Jatrorrhizine (**111**)



Dauricine (**112**)



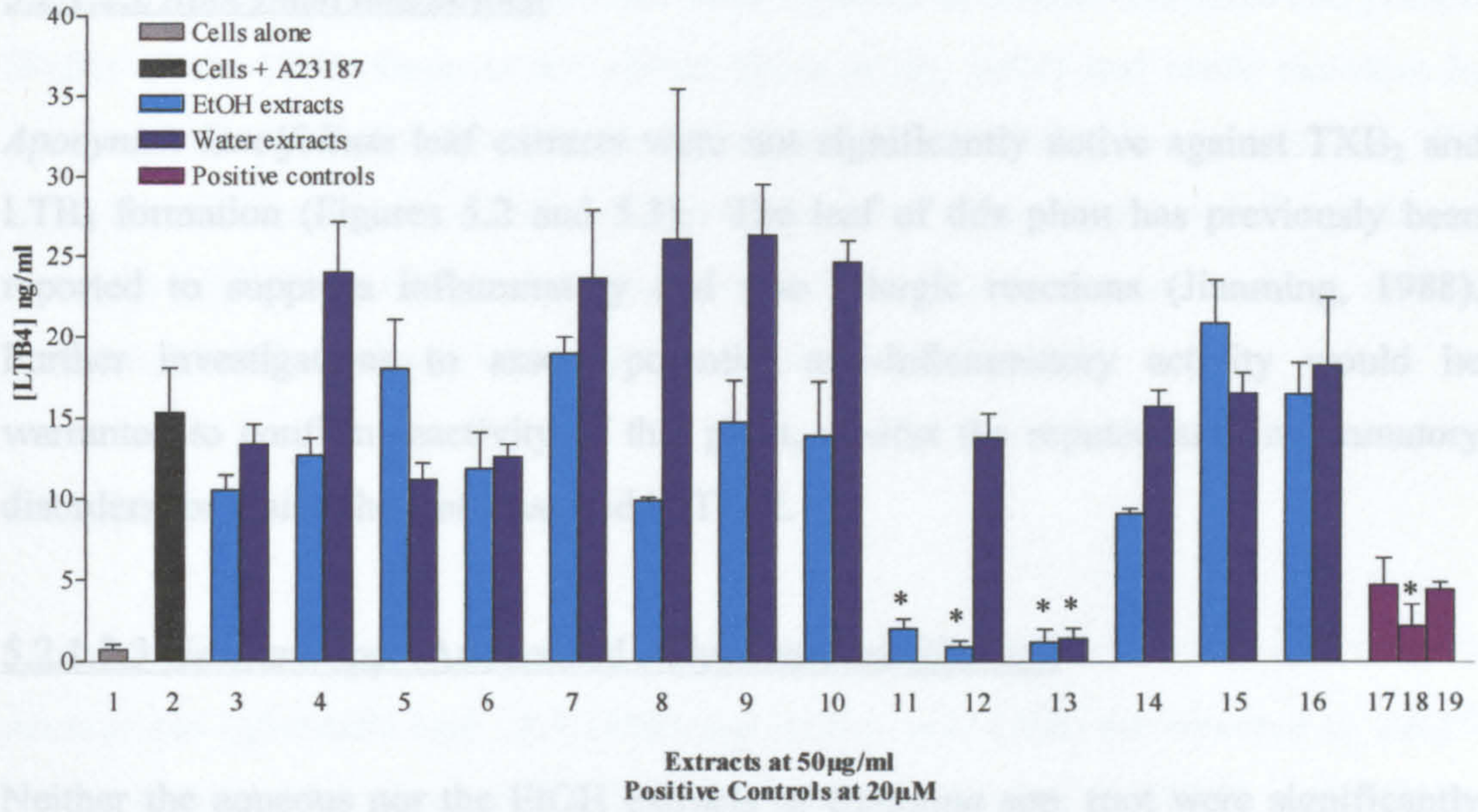
Cryogenine (**113**)

5.2.1.2 Activity of Plant Extracts Against Leukocyte LTB_4 Formation

The aqueous and EtOH plant extracts were also investigated at a concentration of 50µg/ml to determine their activity against leukocyte LTB_4 formation (Figure 5.3). Activation by ionophore A23187 of the peritoneal leukocytes caused a 21-fold increase in LTB_4 generation, which was significantly reduced by 87%, 94%, 92% and 91% by the EtOH extracts of *Rosmarinus officinalis* leaf (fresh), *Rosmarinus officinalis* leaf (dried), *Salvia miltiorrhiza* root and the aqueous extract of *Salvia miltiorrhiza* root respectively ($p<0.05$) (Figure 5.3).

5.2.1.2.1 *Alisma orientalis*

Both the aqueous and EtOH extracts of *Alisma orientalis* root showed no significant activity against LTB_4 or TXB_2 generation (Figures 5.2 and 5.3), suggesting absence of compounds that inhibit the COX and 5-LOX pathways. *A. orientalis* root has previously been reported to suppress xylene-induced mouse ear oedema (Yue *et al.*, 1991), which was attributed to an anti-inflammatory effect. Activity *in vivo* may have been observed following metabolism of inactive compounds in the extract to compounds that are active against enzymes of the arachidonate pathways, or may have occurred independently from effects on TXB_2 or LTB_4 generation. These effects may explain the inactivity of *A. orientalis* against eicosanoid generation *in vitro*.



Key

1: Cells alone (C1)

2: Cells + A23187 (C2 - C6)

3: *Alisma orientalis* root

4: *Apocynum lancifolium* leaf

5: *Centella asiatica* leaf

6: *Codonopsis pilulosa* root

7: *Convallaria majalis* leaf

8: *Melissa officinalis* leaf

9: *Polygala tenuifolia* root

10: Adulterated *Polygonum multiflorum* root (*Gentiana* spp.)

11: *Rosmarinus officinalis* fresh leaf

12: *Rosmarinus officinalis* dried leaf

13: *Salvia miltiorrhiza* root

14: *Withania somnifera* root

15: *Ziziphus jujuba* seed

16: *Ziziphus jujuba* var *spinosa* seed

17: Indomethacin (C7)

18: ZM 230487 (C8)

19: ZM 211965 (C9)

* $p<0.05$; ** $p<0.01$; *** $p<0.001$

Figure 5.3. Effect of aqueous and ethanolic plant extracts and three reference compounds on LTB_4 generation in rat peritoneal leukocytes (n=3, except cells alone: n=6, and A23187 controls: n=24).

5.2.1.2.2 *Apocynum lancifolium*

Apocynum lancifolium leaf extracts were not significantly active against TXB_2 and LTB_4 formation (Figures 5.2 and 5.3). The leaf of this plant has previously been reported to suppress inflammatory and also allergic reactions (Jianming, 1988). Further investigations to assess potential anti-inflammatory activity would be warranted to confirm inactivity of this plant, against the reputed anti-inflammatory disorders for which the leaf was used in TCM.

5.2.1.2.3 *Gentiana* spp. (Adulterated *Polygonum multiflorum*)

Neither the aqueous nor the EtOH extracts of *Gentiana* spp. root were significantly active against TXB_2 or LTB_4 formation *in vitro*, but the EtOH extract was more active against eicosanoid formation than the aqueous extract (Figures 5.2 and 5.3). These results indicate that less polar compounds in the root may be more active against eicosanoid generation than the more polar compounds.

Constituents isolated from *Gentiana* spp. include flavonoids (e.g. isovitexin, quercetin (**104**)) (Lin *et al.*, 1997), secoiridoids (e.g. amarogentin, gentiopicroside, swertiamarine) (Chueh *et al.*, 2001; Tan *et al.*, 1997; Verotta, 1985) and xanthones (e.g. bellidfolin, neolancerin, swerchirin) (Schaufelberger and Hostettmann, 1988). Some flavonoid compounds are reported to have anti-inflammatory activity (de la Puerta *et al.*, 1999; Mower *et al.*, 1984; Welton *et al.*, 1986), but the identification and pharmacological activities of any flavonoids, or other compounds, present in the *Gentiana* spp. used in the present study remain to be further investigated.

5.2.1.2.4 *Polygala tenuifolia*

Polygala tenuifolia extracts were not significantly active in these assays. *P. tenuifolia* has not been reported to date to have anti-inflammatory activity. However, a species related to *P. tenuifolia*, *Polygala cyparissias*, antagonised the contractions induced by chemical inflammatory mediators in the guinea-pig trachea *in vitro*, and this effect was also observed with the isolated compound 1, 7-dihydroxy-2, 3-dimethoxy xanthone (ElSayah *et al.*, 1999). It would be of interest to assess *Polygala cyparissias* for activity against eicosanoid generation to gain further information regarding its

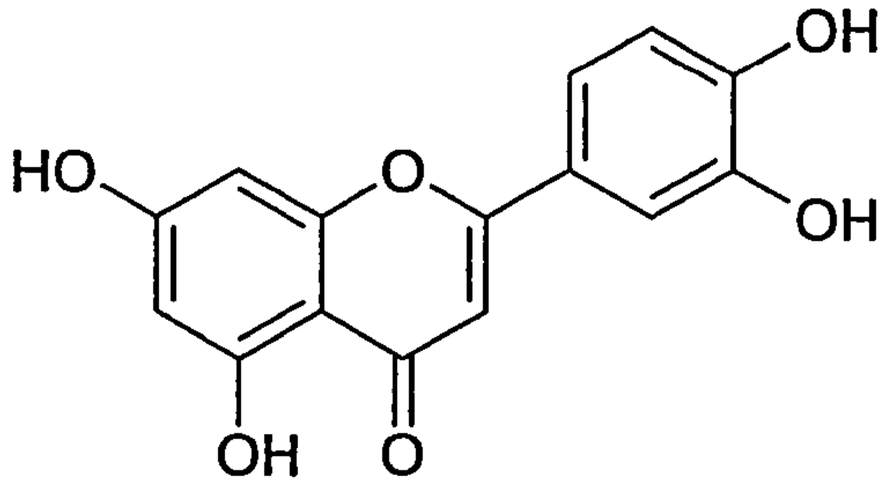
mode of action. *P. tenuifolia* has also been reported to contain xanthone compounds (Fujita *et al.*, 1992; Ikeya *et al.*, 1991a; Ikeya *et al.*, 1994) and could therefore be assessed and compared for the activity observed with *Polygala cyparissias*. The apparent differences in activity observed between these two related species of *Polygala* emphasises the need to assess extracts and isolated compounds in various assays to identify modes of action, potency and potential toxicity.

5.2.1.2.5 *Rosmarinus officinalis*

The phenolic compounds RA (**108**) and caffeic acid (**106**) have been identified in *Rosmarinus officinalis* leaf. RA (**108**) and caffeic acid (**106**) are reported to inhibit the formation of 5-hydroxy-6, 8, 11, 14-eicosatetraenoic acid (5-HETE) and LTB_4 in human polymorphonuclear leucocytes (Kimura *et al.*, 1987). RA (**108**) and caffeic acid (**106**) may have been responsible for the observed activity to reduce LTB_4 formation. However, the occurrence of other active compounds in the extract cannot be excluded. It is therefore possible that RA (**108**), and perhaps other compounds (e.g. other monocaffeoyl derivatives), present in the EtOH extract of *R. officinalis* leaf, are 5-LOX inhibitors. The phenylpropanoid compounds RA (**108**) and caffeic acid (**106**) are relatively hydrophobic in nature, so their presence in the active EtOH extract rather than the less active aqueous extract may be expected. The phenylpropanoid anethole (**99**), present in anise oil, also blocks inflammation, an effect attributed to inhibition of NF-κB activation (Chainy *et al.*, 2000).

The flavonoid components of *R. officinalis* leaf may also be responsible for 5-LOX (and also COX) inhibition. It has been shown that flavones and flavanones with 3, 7, 4-hydroxyl substituents are potent inhibitors of 5-LOX, and the presence of hydroxyl at the 5 position is reported to increase the activity (Welton *et al.*, 1986). The hydroxyl substituents on the flavone luteolin (**114**), which is present in *R. officinalis* leaf, are at the 5, 7, 3 and 4 positions, which would favour 5-LOX inhibition. These structural features required for 5-LOX selectivity were also confirmed by analysis of a flavonoid glycoside and related aglycones (Moroney *et al.*, 1988). It has also been suggested that flavonoid glycosides are weaker inhibitors of 5-LOX than the corresponding aglycones (Moroney *et al.*, 1988); this may explain why the EtOH extract of *R. officinalis* (more likely to contain flavonoid aglycones) was more active against 5-LOX activity than the aqueous extract (more likely to contain flavonoid

glycosides). Flavonoids isolated from other Labiates, *Stachys chrysantha* and *Stachys candida*, inhibit LTC_4 release from mouse peritoneal platelets and TXB_2 release from human platelets (Skaltsa *et al.*, 2000). Flavonoids may also influence phospholipase A_2 activity (Welton *et al.*, 1986), which may also explain activity of the EtOH extract of *R. officinalis* leaf against both COX and 5-LOX.



Luteolin (**114**)

5.2.1.3 Activity of *Salvia miltiorrhiza* Root Extracts Against Leukocyte Eicosanoid Formation

The EtOH extract of *S. miltiorrhiza* root was highly active against both TXB_2 and LTB_4 generation over the dose range 25µg/ml - 200µg/ml (Figures 5.4 and 5.5). This effect may be due to the presence of both COX and 5-LOX inhibitors in the extract, or due to the presence of constituents inhibiting phospholipase A_2 in the inflammatory pathway.

Several other *Salvia* spp. and their constituents are reputed to have anti-inflammatory effects, such as *S. chinensis*, *S. lavandulaefolia*, *S. officinalis* and *S. transsylvancia* (Bartram, 1995; Bisset, 1994; Duke, 1985; Duke and Ayensu, 1985; Maklad *et al.*, 1999). *S. plebeia* is used in Taiwan to treat inflammatory disorders; activity being attributed to naphthoquinone derivatives which have a similar pharmacological profile to NSAIDs (Hernandez-Perez *et al.*, 1995). Anti-inflammatory constituents isolated from *Salvia* spp. include flavonoids (e.g. cirsiliol), monoterpenoids (e.g. carvacrol (**147**), 1, 8-cineole (**56**), thymol) and phenylpropanoids (e.g. caffeic acid (**106**), eugenol (**84**), RA (**108**)) (Kuhnt *et al.*, 1995; Marder *et al.*, 1996; Santos and Rao, 2000; Wagner *et al.*, 1986).

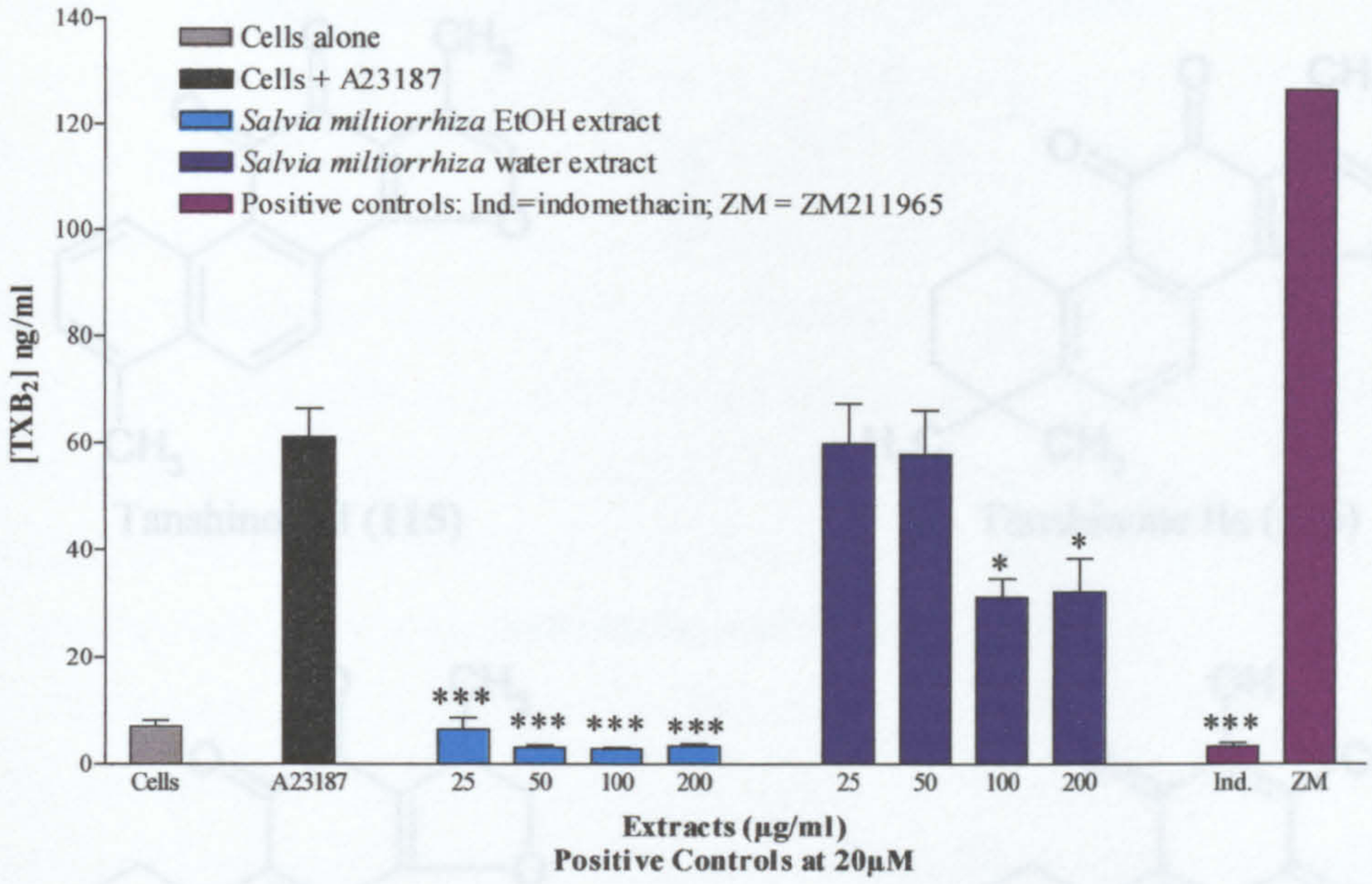


Figure 5.4. Effect of *Salvia miltiorrhiza* root extracts and two reference compounds on TXB_2 generation in rat peritoneal leukocytes (n=3, except cells alone: n=6, and A23187 controls: n=24).

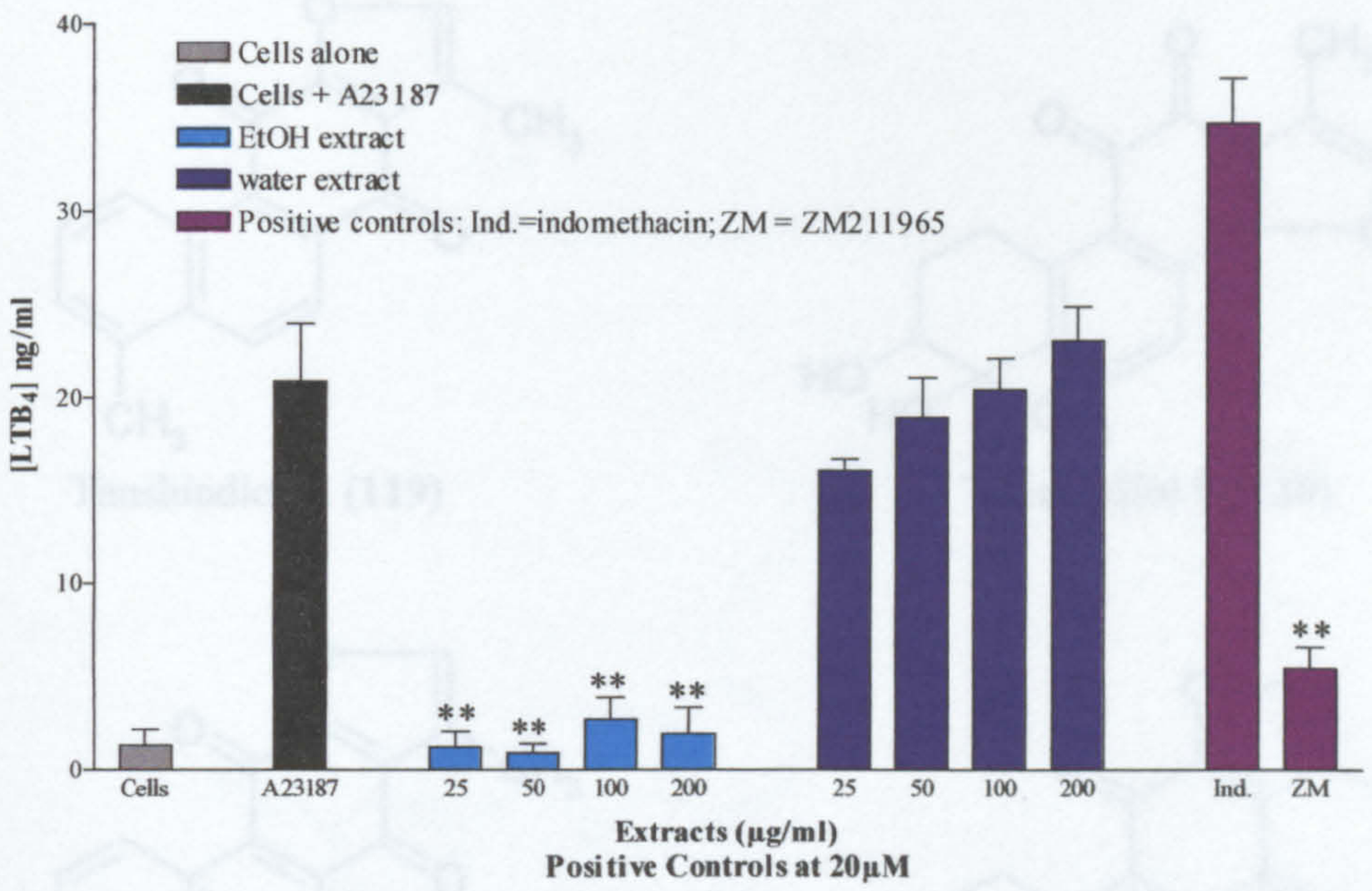
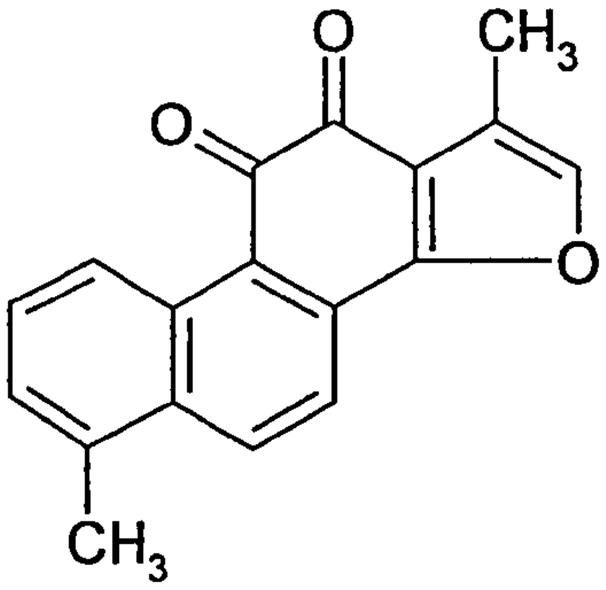
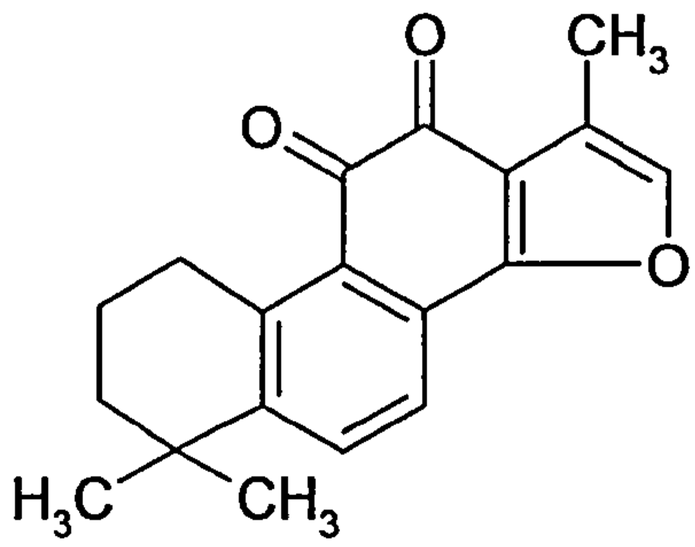


Figure 5.5. Effect of *Salvia miltiorrhiza* root extracts and two reference compounds on LTB_4 generation in rat peritoneal leukocytes (n=3, except cells alone: n=6, and A23187 controls: n=24).

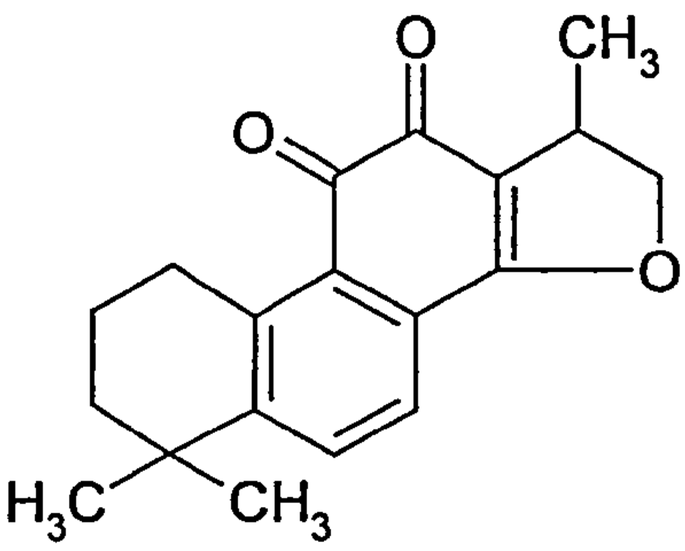
* $p<0.05$; ** $p<0.01$; *** $p<0.001$



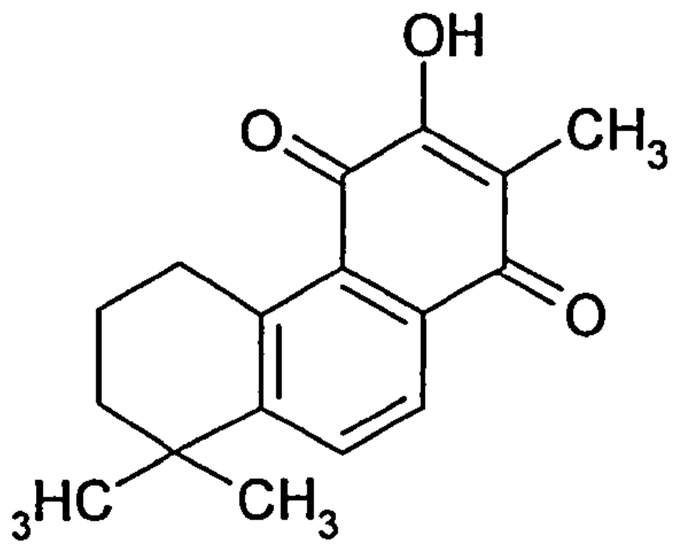
Tanshinone I (**115**)



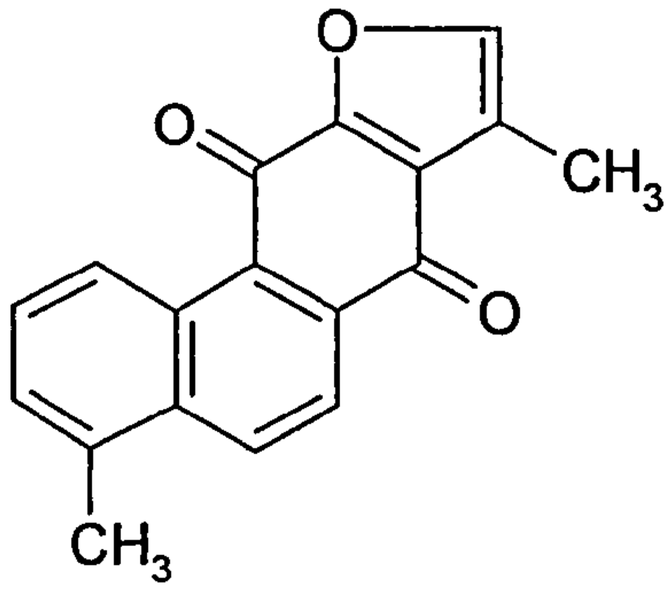
Tanshinone IIa (**116**)



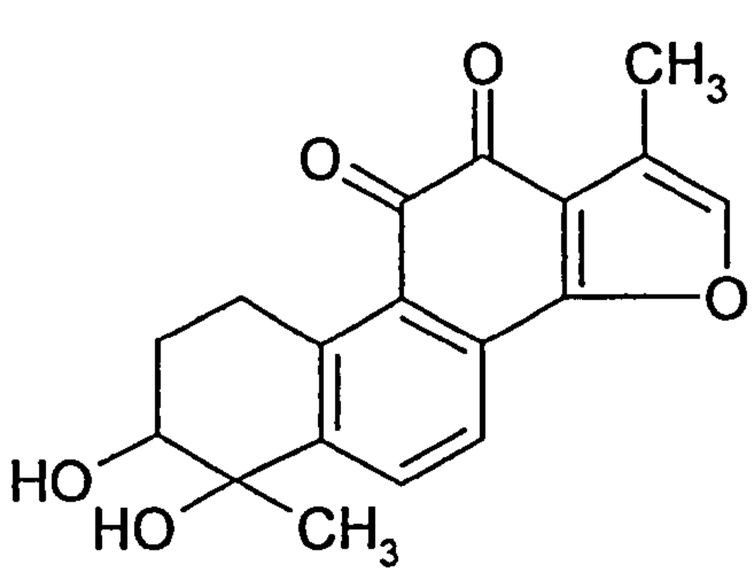
Cryptotanshinone (**117**)



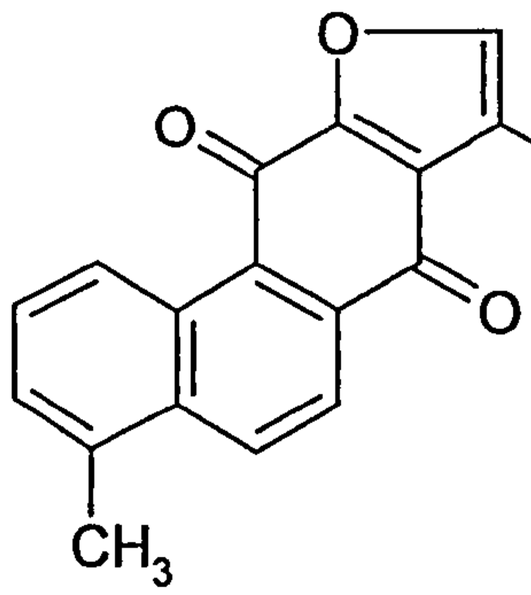
Neocryptotanshinone II (**118**)



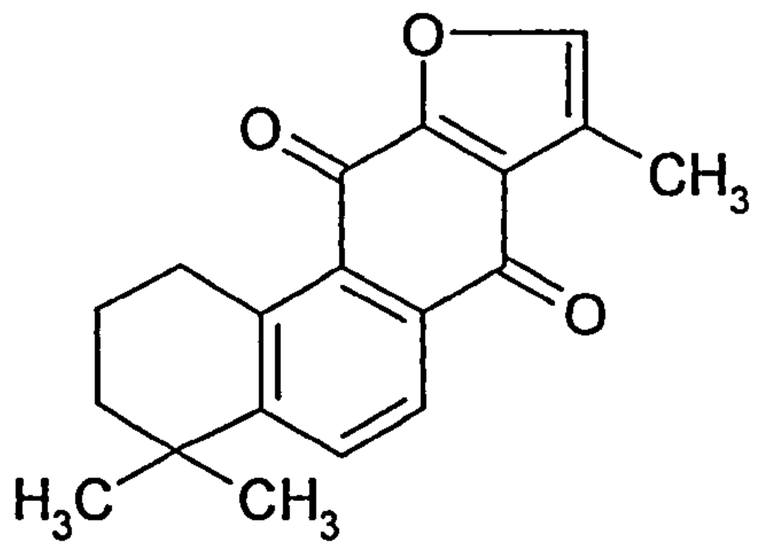
Tanshindiol A (**119**)



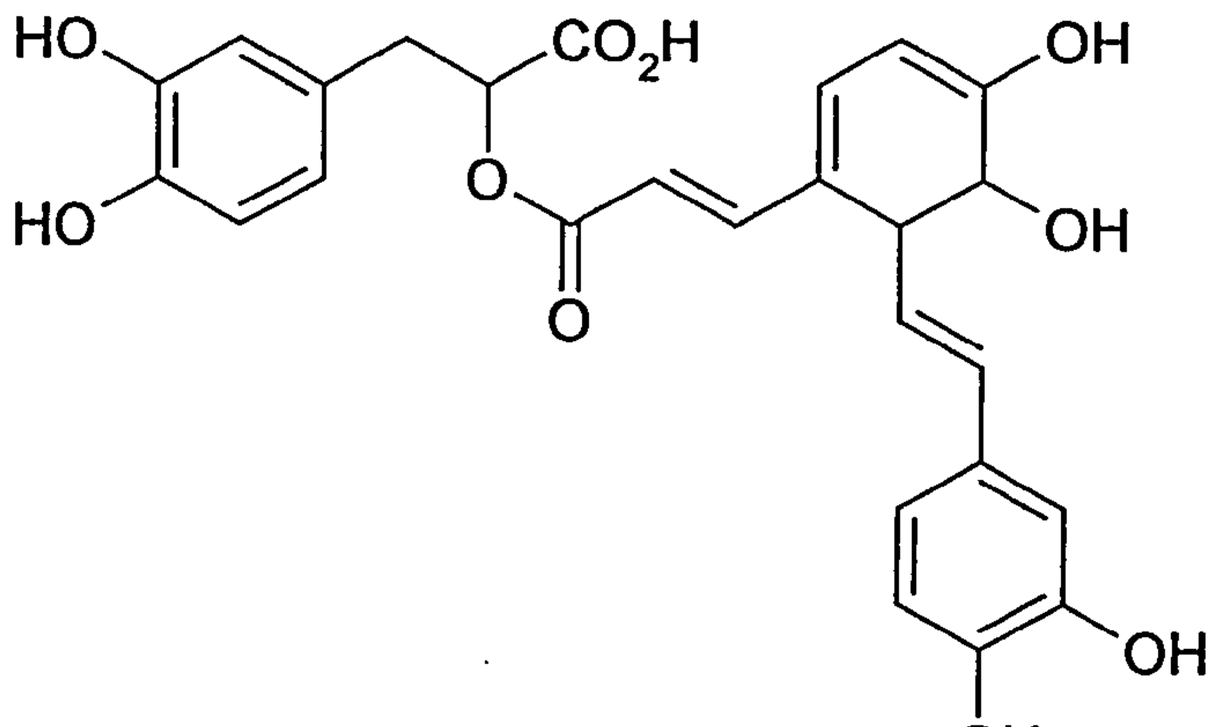
Tanshindiol B (**120**)



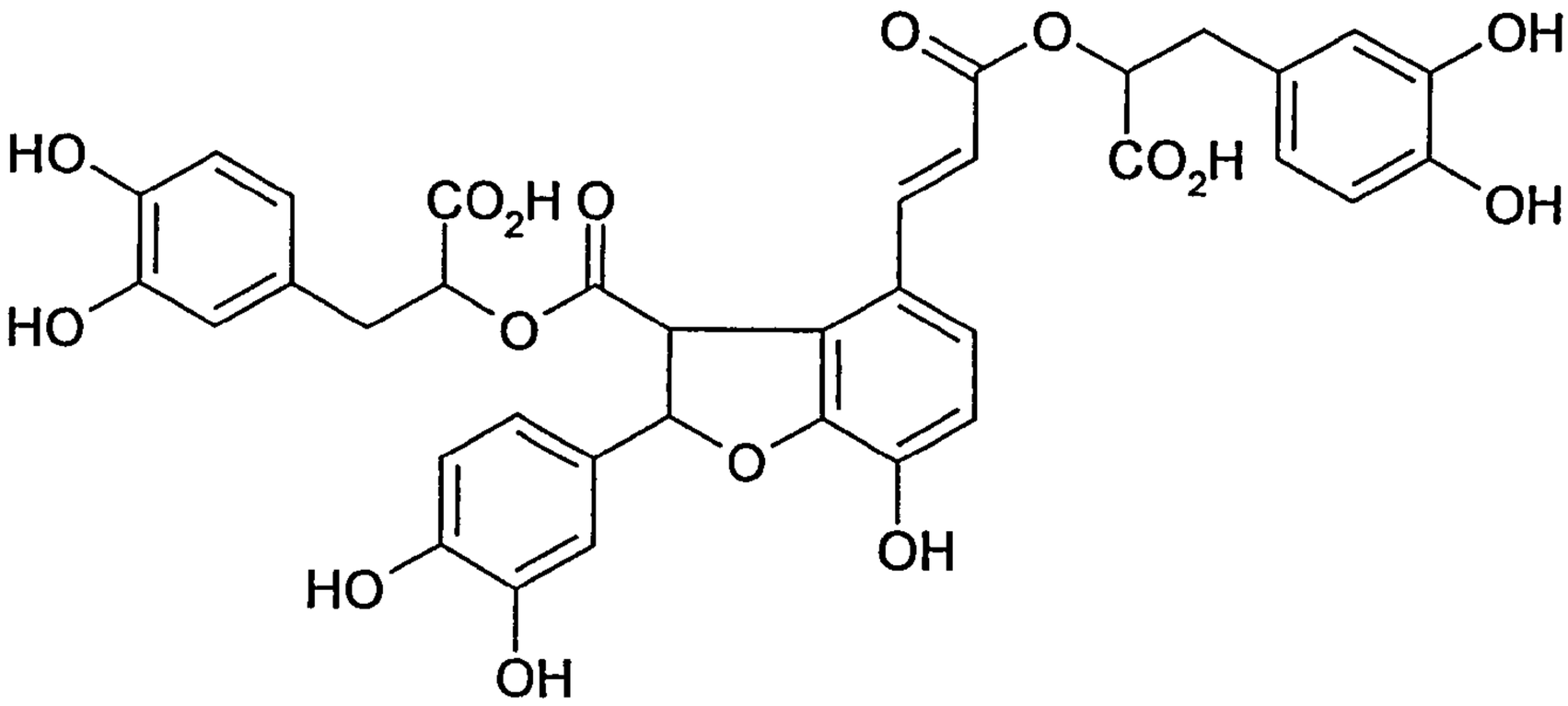
Isotanshinone I (**121**)



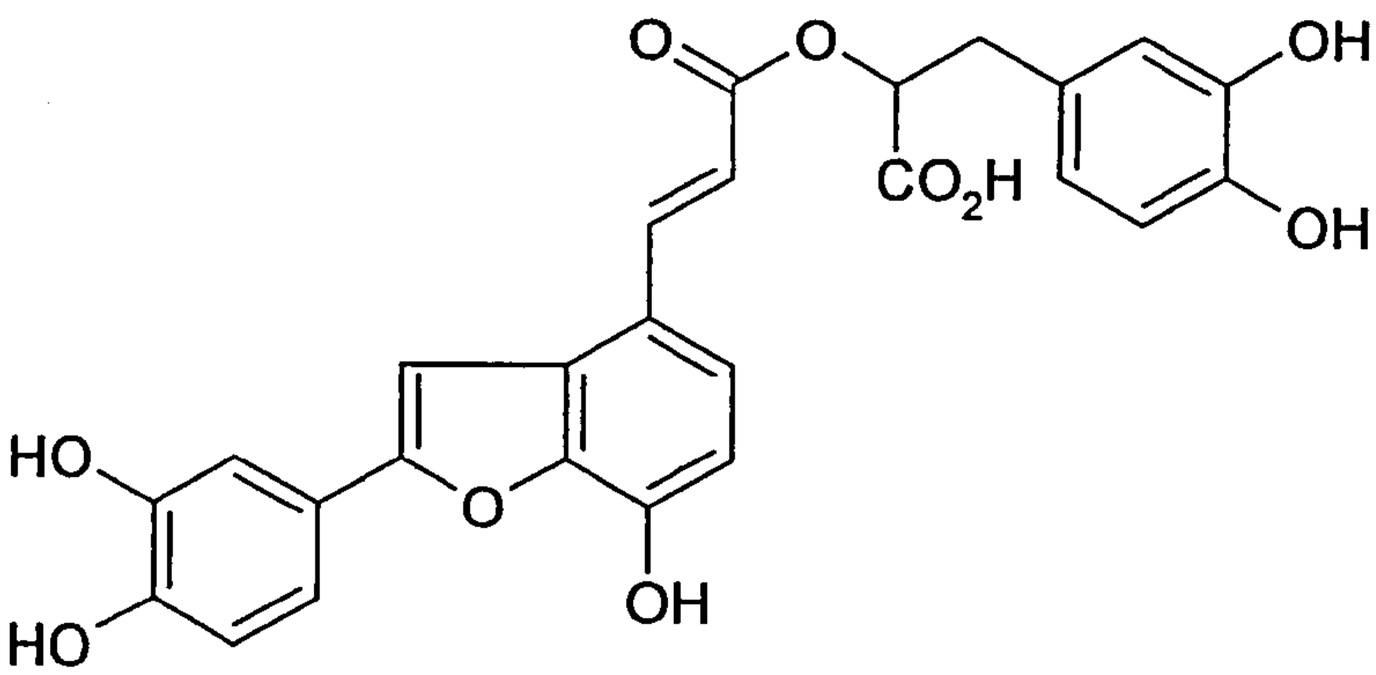
Isotanshinone II (**122**)



Salvianolic acid A (**123**)



Salvianolic acid B (**124**)

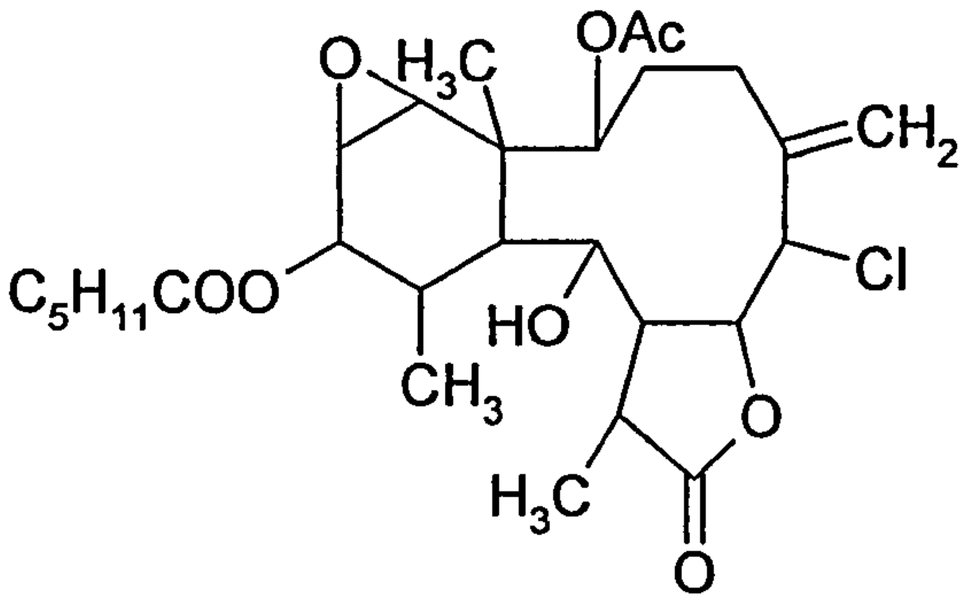


Salvianolic acid C (**125**)

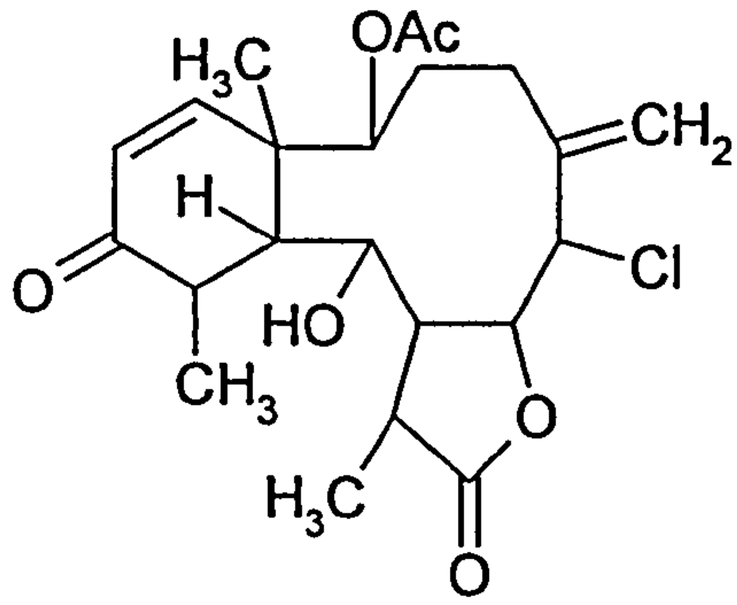
Compounds isolated from *S. miltiorrhiza* root include several diterpenoid compounds such as tanshinones I (**115**) and IIa (**116**), cryptotanshinone (**117**), tanshindiols A (**119**) and B (**120**), isotanshinones I (**121**) and II (**122**) and neocryptotanshinone II (**118**) (Lin and Chang, 2000; Tang and Eisenbrand, 1992). Several water-soluble compounds have been isolated from the aqueous extract of *S. miltiorrhiza* root, including danshensu (**45**) (Chen and Liu, 1980), the salvianolic acids A (**123**) (Li *et al.*, 1984), B (**124**) and C (**125**) (Ai and Li, 1988) and RA (Huang *et al.*, 1992).

Tanshinones are reported to be anti-inflammatory in rats with infective arthritis (Duke and Ayensu, 1985) and in mice with croton oil induced inflammation of the ear (Tang and Eisenbrand, 1992), but the mechanism of action was not established. Dihydrotanshinone and cryptotanshinone (**117**) are significant inhibitors of β-hexosaminidase release from mast cells *in vitro* (Ryu *et al.*, 1999), which indicates they may have anti-allergic activity. Tanshinones I (**115**) and IIa (**116**), cryptotanshinone (**117**) and dihydrotanshinone have also been identified as platelet aggregation inhibitors, suggesting a potential anti-inflammatory action (Onitsuka, M. *et al.*, 1983).

Many diterpenoid compounds have demonstrated anti-inflammatory activity from both plant and marine sources. These include the solenolide diterpenes from marine *Solenopodium* spp.; solenolide A (**126**) is an inhibitor of 5-LOX and solenolide E (**127**) is an inhibitor of COX (Harborne and Tomas-Barberan, 1991). Diterpenes isolated from the genus *Sideritis* (also a Labiate) are anti-inflammatory in several animal models, such as the diterpene borjatriol (Alcaraz and Villar, 1987; Villar *et al.*, 1983; Villar *et al.*, 1984); the two diterpenes (*ent*-13-*epi*-12α-acetoxymanoyl oxide and *ent*-8α-hydroxy-labda-13(16), 14-diene) isolated from *Sideritis javalambrensis* inhibited PGE_2 generation in cultured mouse peritoneal macrophages (de las Heras and Hoult, 1994).



Solenolide A (**126**)



Solenolide E (**127**)

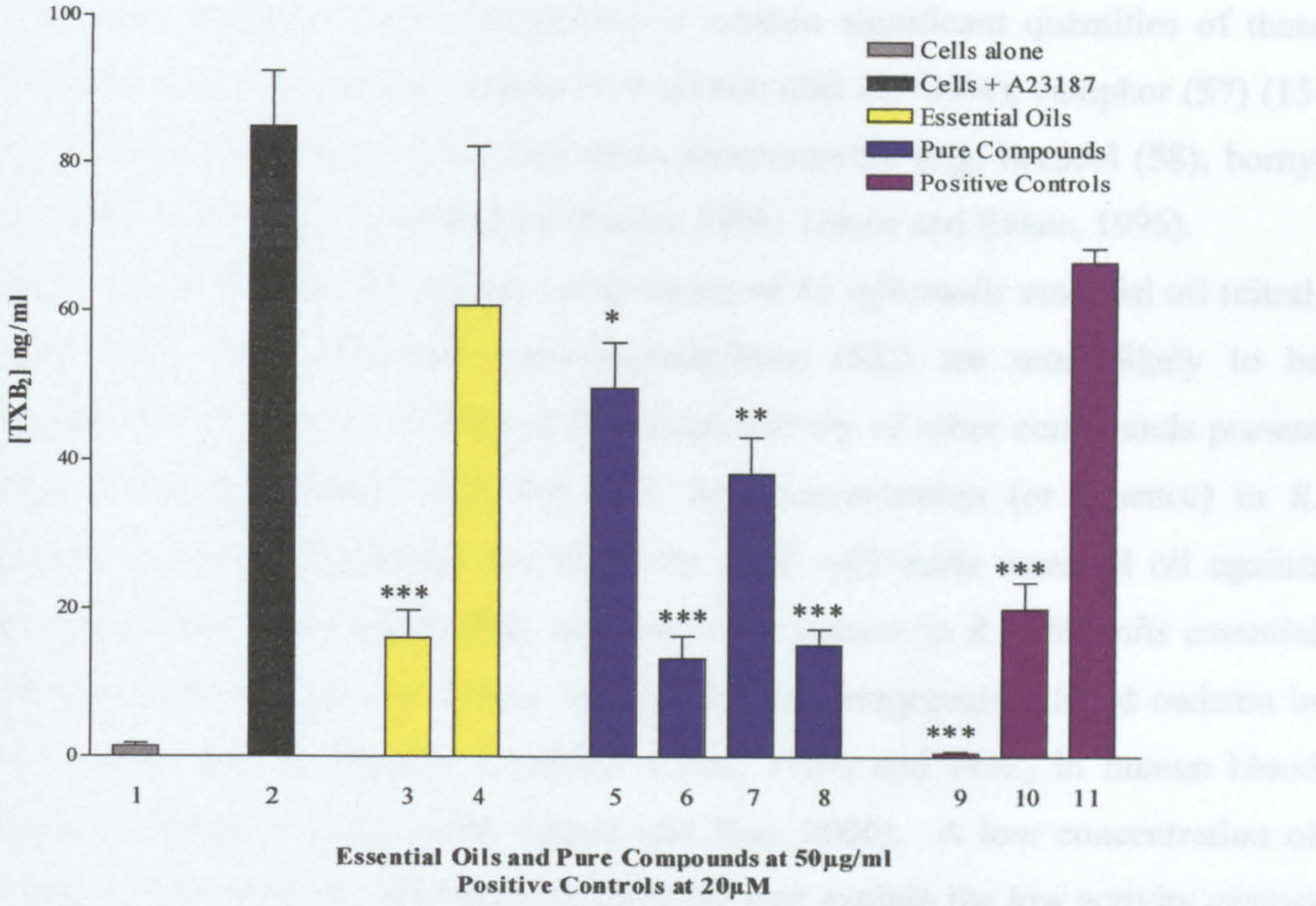
It is therefore likely that the diterpenes present in *S. miltiorrhiza* root are responsible for the activity against eicosanoid generation, although other compounds present may also contribute to activity, perhaps synergistically. Recent investigations support the findings of these studies. Tanshinone I (**115**), dihydrotanshinone, tanshinone IIa (**116**) and cryptotanshinone (**117**) isolated from the lipophilic extracts of *S. miltiorrhiza* root demonstrated activity against 5-LOX in porcine leukocytes, but were not as active as the crude extracts (Paulus and Bauer, 2000). This confirms that less polar extracts (which include diterpene components) are more active than the more polar, aqueous extract, and although diterpenes may contribute to activity, other compounds or synergism may explain the higher potency observed with the crude EtOH extract. Therefore, isolation of compounds responsible for COX inhibition, and continued assessment of 5-LOX inhibitors, is required.

5.2.2 Activity of Essential Oils and Essential Oil Constituents Against Leukocyte Eicosanoid Formation

5.2.2.1 Activity of Essential Oils and Essential Oil Constituents Against Leukocyte TXB_2 Formation

The essential oils from *Rosmarinus officinalis* and *Melissa officinalis* and the essential oil constituents *trans*-caryophyllene (**82**), citral, geraniol (**72**) and nerol (**75**), the major constituents present in *M. officinalis* essential oil (refer to Chapter 2, 2.2.7.1), were investigated at a concentration of 50µg/ml to determine their activity against leukocyte TXB_2 formation. The results show that *M. officinalis* essential oil, citral and nerol (**75**) were significantly active against TXB_2 formation ($p<0.001$) (Figure 5.6).

Activation by ionophore A23187 of the peritoneal leukocytes caused a 63-fold increase in TXB_2 generation, which was reduced by 81%. 84% and 82% by *M. officinalis* essential oil, citral and nerol (**75**) respectively ($p<0.001$). Geraniol (**72**) and *trans*-caryophyllene (**82**) were also significantly active against TXB_2 formation ($p<0.01$ and $p<0.005$ respectively) (Figure 5.6). The terpenes citral, geraniol (**72**), nerol (**75**) and *trans*-caryophyllene (**82**) comprise 42% of the *M. officinalis* essential oil tested (refer to Chapter 2, 2.2.7.1).



Key

1: Cells alone (C1)

2: Cells+A23187 (C2-C6)

3: *M. officinalis* essential oil

4: *R. officinalis* essential oil

5: *Trans*-caryophyllene

6: Citral

7: Geraniol

8: Nerol

9: Indomethacin (C7)

10: ZM 230487 (C8)

11: ZM 211965 (C9)

Figure 5.6. Effect of essential oils, essential oil constituents and three reference compounds on TXB_2 generation in rat peritoneal leukocytes (n=3, except cells alone: n=6 and A23187 controls: n=24).

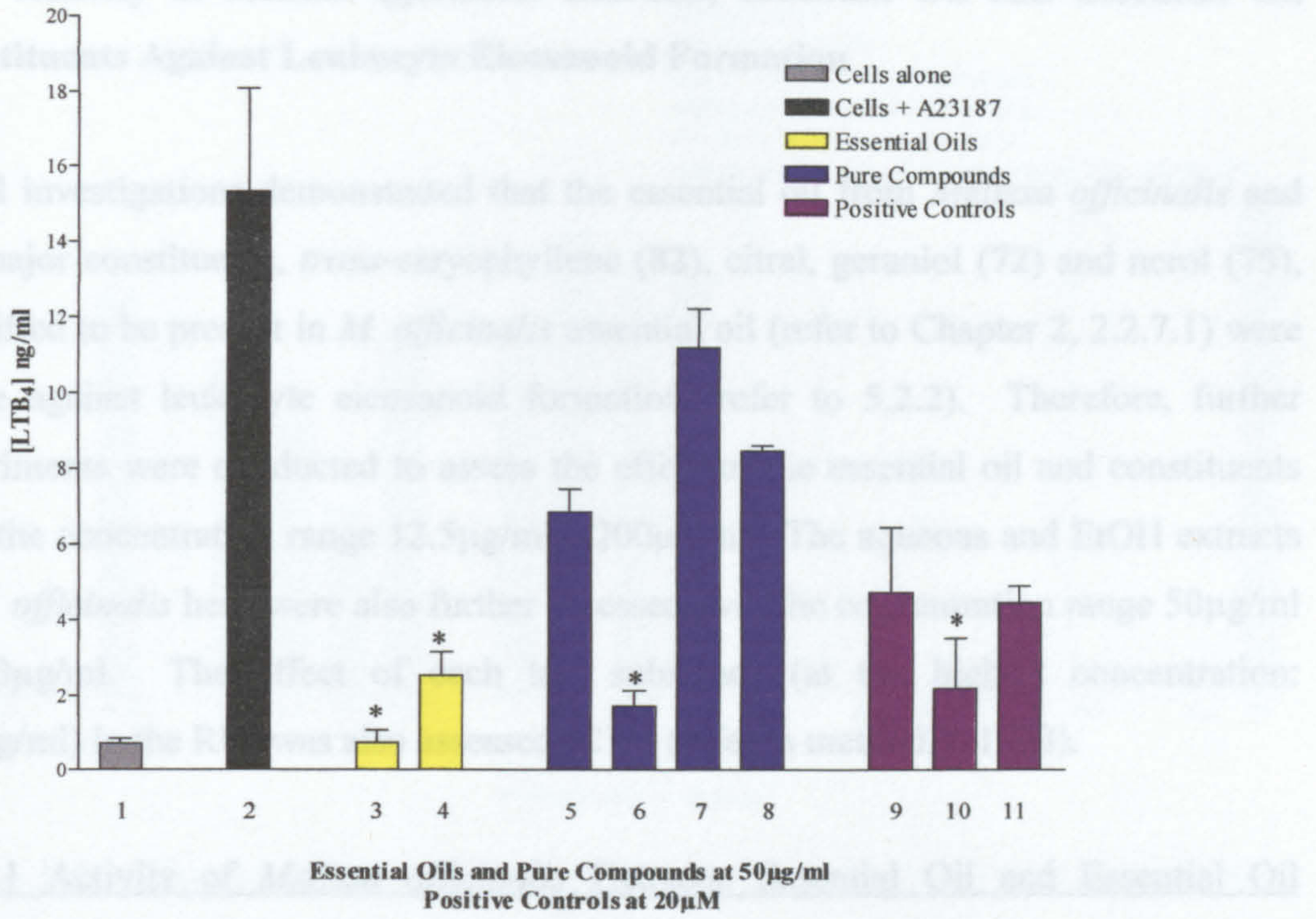
* $p<0.05$; ** $p<0.01$; *** $p<0.001$

R. officinalis essential oil is not reported to contain significant quantities of these compounds, but is reported to contain 1, 8-cineole (**56**) (15-30%), camphor (**57**) (15-25%), α-pinene (**53**) (up to 25%) and other monoterpenes (e.g. borneol (**58**), bornyl acetate (**51**)) as the major constituents (Bisset, 1994; Trease and Evans, 1996).

It may be concluded that the terpene components of *M. officinalis* essential oil (citral, geraniol (**72**), nerol (**75**) and *trans*-caryophyllene (**82**)) are most likely to be responsible for the activity of this oil (although activity of other compounds present remains to be determined), and that their low concentration (or absence) in *R. officinalis* essential oil explains the inactivity of *R. officinalis* essential oil against TXB_2 generation. 1, 8-cineole (**56**), reported to be present in *R. officinalis* essential oil (Bisset, 1994; Trease and Evans, 1996), inhibits carrageenan-induced oedema in paws of mice, and is reported to inhibit LTB_4, TXB_2 and PGE_2 in human blood monocytes (Juergans *et al.*, 1998; Santos and Rao, 2000). A low concentration of 1, 8-cineole (**56**) in the *R. officinalis* essential oil may explain the low activity against TXB_2 generation in the rat peritoneal leukocytes.

5.2.2.2 Activity of Essential Oils and Essential Oil Constituents Against Leukocyte LTB_4 Formation

The essential oils from *Rosmarinus officinalis* and *Melissa officinalis*, and the essential oil constituents *trans*-caryophyllene (**82**), citral, geraniol (**72**) and nerol (**75**) were also investigated at a concentration of 50µg/ml to determine their activity against leukocyte LTB_4 formation. The results show that *R. officinalis*, *M. officinalis* and citral were active against LTB4 formation ($p<0.05$) (Figure 5.7). These results indicate the presence of 5-LOX inhibitors in both essential oils. Different compounds in each essential oil may be responsible for activity, as the oil compositions differ. The presence of citral in *M. officinalis* essential oil may have contributed to activity, but other constituents may have also influenced LTB4 generation, perhaps synergistically.



Key

1: Cells alone (C1)

2: Cells+A23187 (C2-C6)

3: *M. officinalis* essential oil

4: *R. officinalis* essential oil

5: *Trans*-caryophyllene

6: Citral

7: Geraniol

8: Nerol

9: Indomethacin (C7)

10: ZM 230487 (C8)

11: ZM 211965 (C9)

Figure 5.7. Effect of essential oils, essential oil constituents and three reference compounds on LTB_4 generation in rat peritoneal leukocytes (n=3, except cells alone: n=6 and A23187 controls: n=24).

* $p<0.05$; ** $p<0.01$; *** $p<0.001$

5.2.3 Activity of *Melissa officinalis* Extracts, Essential Oil and Essential Oil Constituents Against Leukocyte Eicosanoid Formation

Initial investigations demonstrated that the essential oil from *Melissa officinalis* and the major constituents, *trans*-caryophyllene (**82**), citral, geraniol (**72**) and nerol (**75**), identified to be present in *M. officinalis* essential oil (refer to Chapter 2, 2.2.7.1) were active against leukocyte eicosanoid formation (refer to 5.2.2). Therefore, further experiments were conducted to assess the effect of the essential oil and constituents over the concentration range 12.5µg/ml - 200µg/ml. The aqueous and EtOH extracts of *M. officinalis* herb were also further assessed over the concentration range 50µg/ml - 200µg/ml. The effect of each test substance (at the highest concentration: 200µg/ml) in the RIA was also assessed (C11) (refer to method, 5.1.2.3).

5.2.3.1 Activity of *Melissa officinalis* Extracts, Essential Oil and Essential Oil Constituents Against Leukocyte TXB_2 Formation

The EtOH extract of *Melissa officinalis* herb demonstrated concentration dependent activity against leukocyte TXB_2 formation; this activity could not be attributed to interference in the RIA (Figure 5.8). This suggests that compound(s) in the EtOH extract of *M. officinalis* herb may cause COX inhibition. In contrast, the aqueous extract of *M. officinalis* herb was not significantly active against TXB_2 formation over the concentration range tested (Figure 5.8). This suggests that COX inhibitors are absent in this extract. However, COX inhibitors may be present at concentrations too low to cause enzyme inhibition, or both inducers and inhibitors of the COX enzyme may be present, which may also explain inactivity.

M. officinalis herb constituents include RA (**108**), chlorogenic (**107**) and caffeic (**106**) acids, triterpenes and flavonoids (e.g. quercetin (**104**), rhamnocitrin and the glycosides of apigenin and luteolin (**114**)) (Bisset, 1994; Bruneton, 1995; Mulkens and Kapetanidis, 1987). The flavone aglycones apigenin and luteolin (**114**) are reportedly anti-inflammatory (Harborne, 1993), which may explain the activity of the less polar EtOH extract. Other flavonoid compounds may also be responsible for the inhibitory effects against TXB_2 generation observed, but isolation of compounds is necessary to investigate effects of such compounds against TXB_2 formation.

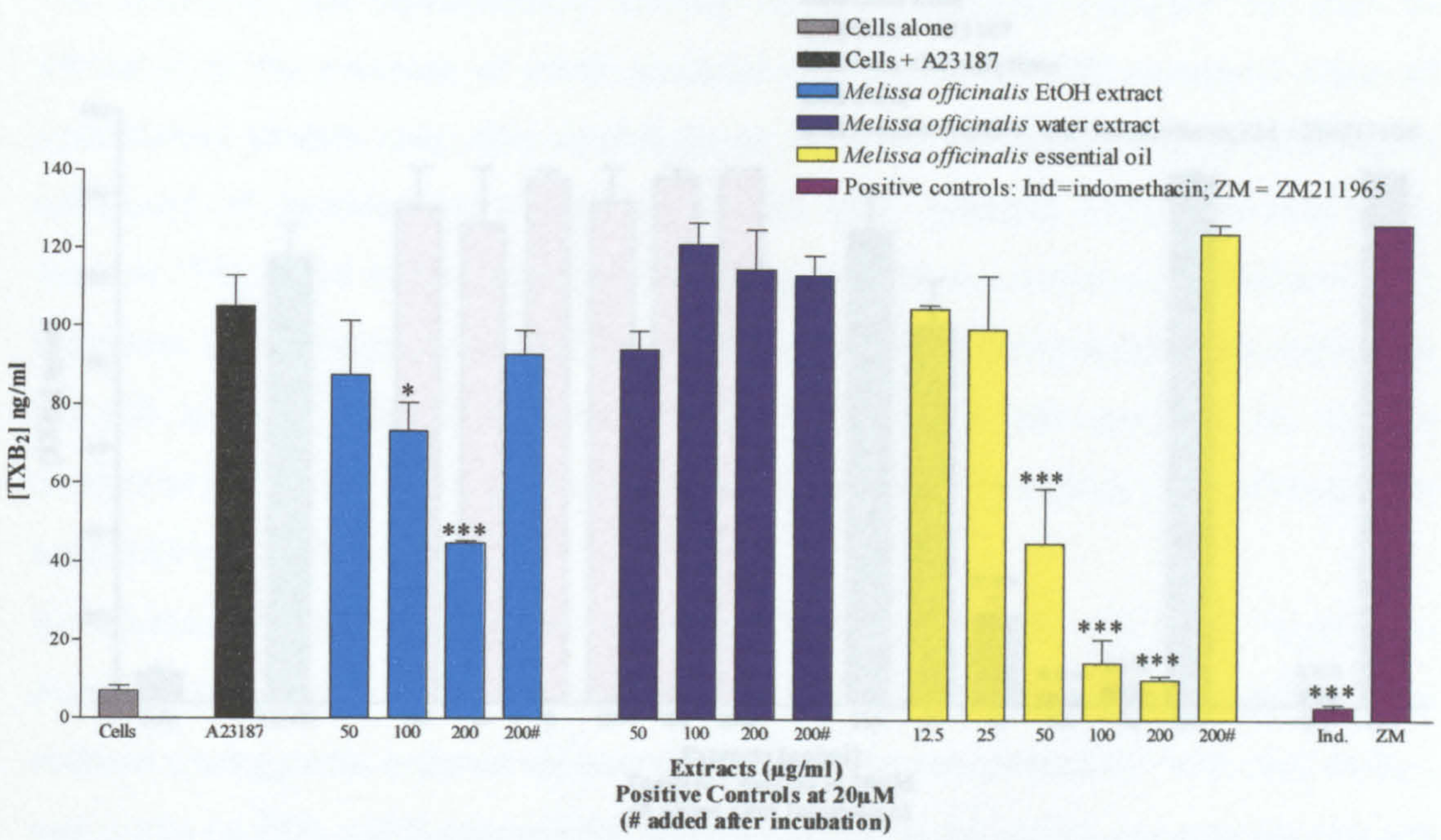


Figure 5.8. Effect of *Melissa officinalis* leaf extracts, essential oil and two reference compounds on TXB_2 generation in rat peritoneal leukocytes (n=3, except cells alone: n=6 and A23187 controls: n=24).

* $p<0.05$; ** $p<0.01$; *** $p<0.001$

M. officinalis essential oil and the pure compounds citral and geraniol (**72**) showed dose dependent activity against TXB_2 formation, and were significantly active over the concentration range 50µg/ml - 200µg/ml ($p<0.001$) (Figures 5.8, 5.9 and 5.10). *Trans*-caryophyllene (**82**) was inactive against TXB_2 formation over the concentration range, which suggests this compound is not a potent COX inhibitor (Figure 5.9). It may therefore be concluded that the presence of *trans*-caryophyllene (**82**) in *M. officinalis* essential oil does not contribute significantly to the activity of the essential oil against TXB_2 formation.

The *cis*-isomer of geraniol (**72**) is nerol (**75**) and it showed significant dose dependent activity against TXB_2 formation over the concentration range 12.5µg/ml - 200µg/ml ($p<0.001$) (Figure 5.10). Neither the essential oil, nor the pure compounds appeared to influence TXB_2 formation as a result of interference in the RIA. Therefore, it is possible that these substances are inhibitors of COX.

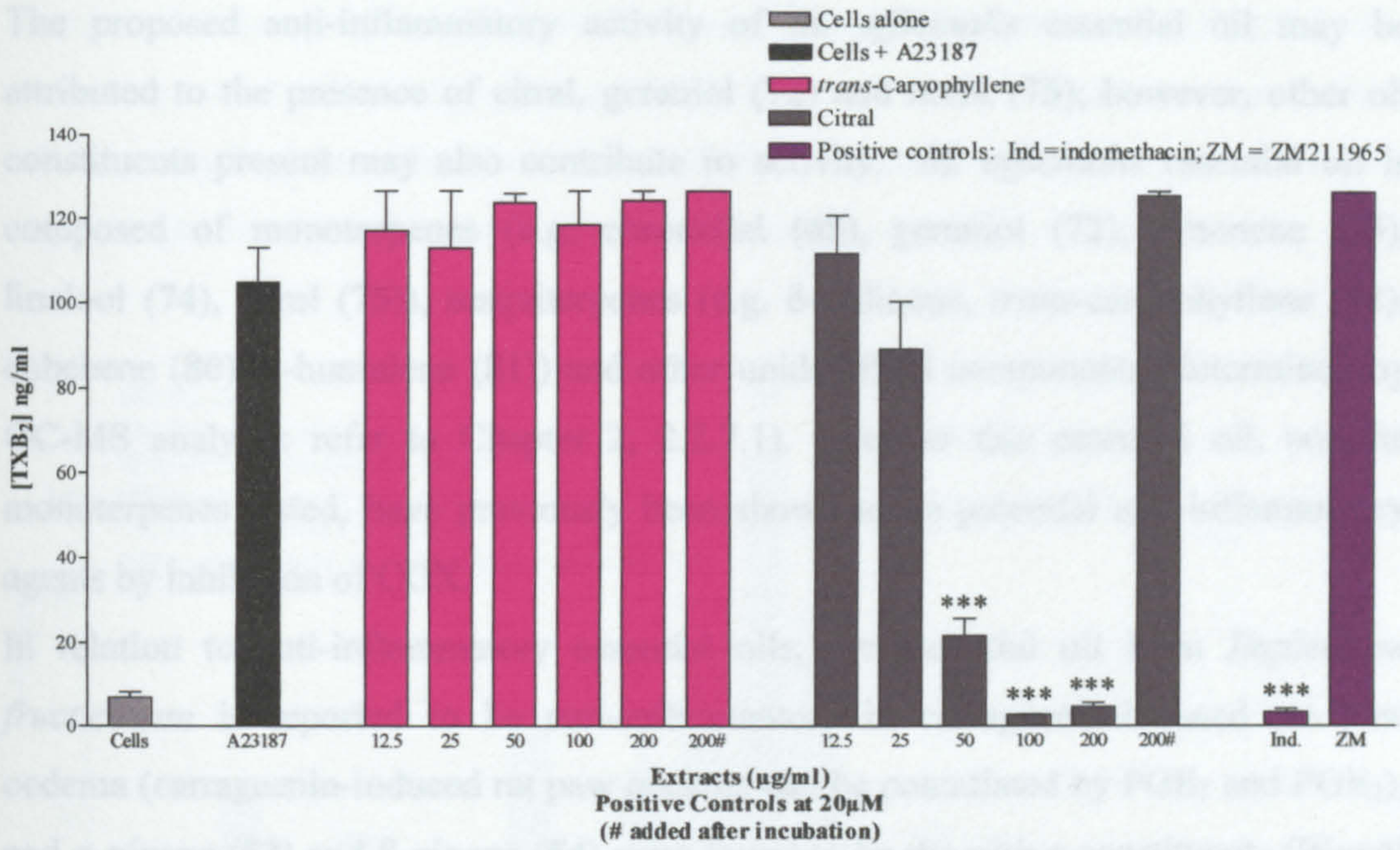


Figure 5.9. Effect of the pure compounds *trans*-caryophyllene and citral, and two reference compounds on TXB_2 generation in rat peritoneal leukocytes (n=3, except cells alone: n=6 and A23187 controls: n=24).

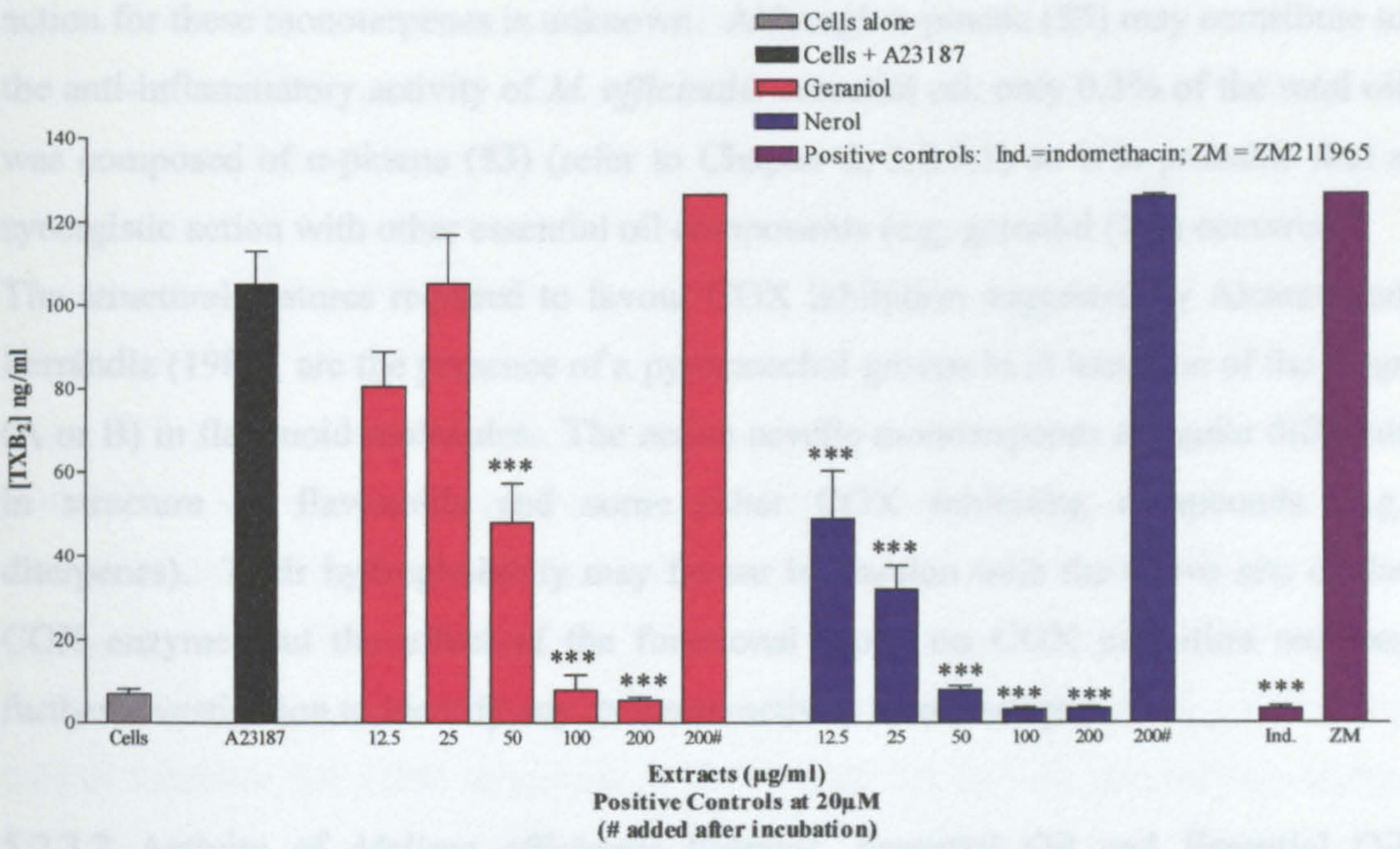


Figure 5.10. Effect of the pure compounds geraniol and nerol, and two reference compounds on TXB_2 generation in rat peritoneal leukocytes (n=3, except cells alone: n=6 and A23187 controls: n=24).

* $p<0.05$; ** $p<0.01$; *** $p<0.001$

The proposed anti-inflammatory activity of *M. officinalis* essential oil may be attributed to the presence of citral, geraniol (**72**) and nerol (**75**); however, other oil constituents present may also contribute to activity. *M. officinalis* essential oil is composed of monoterpenes (e.g. citronellal (**68**), geraniol (**72**), limonene (**73**), linalool (**74**), nerol (**75**)), sesquiterpenes (e.g. δ-cadinene, *trans*-caryophyllene (**82**), cubebene (**80**), α-humulene (**81**)) and other unidentified components (determined by GC-MS analysis: refer to Chapter 2, 2.2.7.1). Neither this essential oil, nor the monoterpenes tested, have previously been shown to be potential anti-inflammatory agents by inhibition of COX.

In relation to anti-inflammatory essential oils, the essential oil from *Bupleurum fructicosum* is reported to be anti-inflammatory in carrageenin-induced rat paw oedema (carrageenin-induced rat paw oedema can be potentiated by PGE_1 and PGE_2), and α-pinene (**53**) and β-pinene (**54**) were found to be the active constituents (Bingöl and Şener, 1995; Handa *et al.*, 1992). (-)-Car-3-ene from *Bupleurum falcatum* essential oil is reported to be more potent than α- (**53**) or β-pinene (**54**) (Bingöl and Şener, 1995; Handa *et al.*, 1992). However, the mechanism of anti-inflammatory action for these monoterpenes is unknown. Although α-pinene (**53**) may contribute to the anti-inflammatory activity of *M. officinalis* essential oil, only 0.3% of the total oil was composed of α-pinene (**53**) (refer to Chapter 2, 2.2.7.1) so it is probable that a synergistic action with other essential oil components (e.g. geraniol (**72**)) occurred.

The structural features required to favour COX inhibition suggested by Alcaraz and Ferrándiz (1987), are the presence of a pyrocatechol groups in at least one of the rings (A or B) in flavonoid molecules. The active acyclic monoterpenes are quite different in structure to flavonoids and some other COX inhibiting compounds (e.g. diterpenes). Their hydrophobicity may favour interaction with the active site of the COX enzyme, but the effect of the functional group on COX inhibition requires further investigation to identify any structure-activity relationships.

5.2.3.2 Activity of *Melissa officinalis* Extracts, Essential Oil and Essential Oil Constituents Against Leukocyte LTB_4 Formation

The EtOH extract of *Melissa officinalis* herb demonstrated significant activity against LTB_4 formation at concentrations 100μg/ml - 200μg/ml (Figure 5.11). The aqueous extract of *M. officinalis* herb was not active against LTB_4 formation over the

concentration range tested (Figure 5.11), and at both 100µg/ml and 200µg/ml, this extract appeared to actually promote LTB_4 formation. This suggests that 5-LOX inducers may be present in this extract, and that inhibitors of 5-LOX are either absent or present in concentrations too low to inhibit enzyme activity.

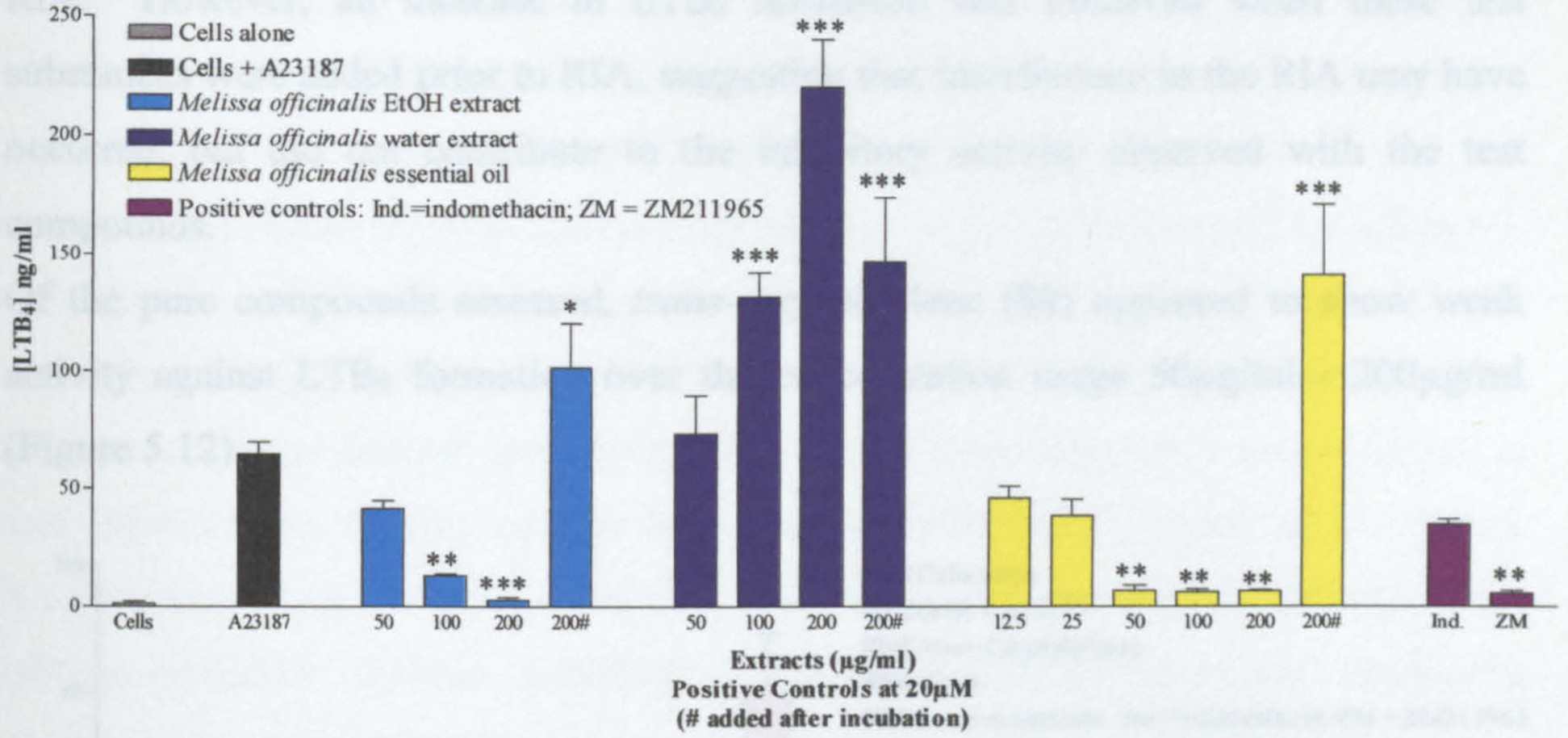


Figure 5.11. Effect of *Melissa officinalis* leaf extracts, essential oil and two reference compounds on LTB_4 generation in rat peritoneal leukocytes (n=3, except cells alone: n=6 and A23187 controls: n=24).

* $p<0.05$; ** $p<0.01$; *** $p<0.001$

The constituent profile of *M. officinalis* leaf is similar to that of *Rosmarinus officinalis* leaf. This may explain the activities observed against eicosanoid generation with the EtOH extracts of both these plants (refer to 5.2.1, Figures 5.2 and 5.3, and 5.2.3, Figures 5.8 and 5.11). However, different percentage compositions of each constituent, and synergistic effects, may influence potency. For example, the known 5-LOX inhibitor RA (**108**) is present in both herbs, but may be present at a higher concentration in *R. officinalis*; this may explain the higher activity against LTB_4 formation, when assayed at 50µg/ml (Figure 5.3). The concentration dependent activity observed with the EtOH extract of *M. officinalis* herb may be due to the presence of RA (**108**) or other monocaffeoyl derivatives, or due to the flavonoid components of the extract and/or other unidentified phytochemicals.

The essential oil from *M. officinalis* was significantly active against LTB_4 formation over the concentration range 50µg/ml - 200µg/ml ($p<0.01$) (Figure 5.11). The inhibition of LTB_4 generation caused by the EtOH extract and the essential oil did not appear to be due to interference in the RIA, as a reduction in LTB_4 formation was not observed when these test substances were added after incubation prior to analysis by RIA. However, an increase in LTB_4 formation was observed when these test substances were added prior to RIA, suggesting that interference in the RIA may have occurred, but did not contribute to the inhibitory activity observed with the test compounds.

Of the pure compounds assessed, *trans*-caryophyllene (**82**) appeared to show weak activity against LTB_4 formation over the concentration range 50µg/ml - 200µg/ml (Figure 5.12).

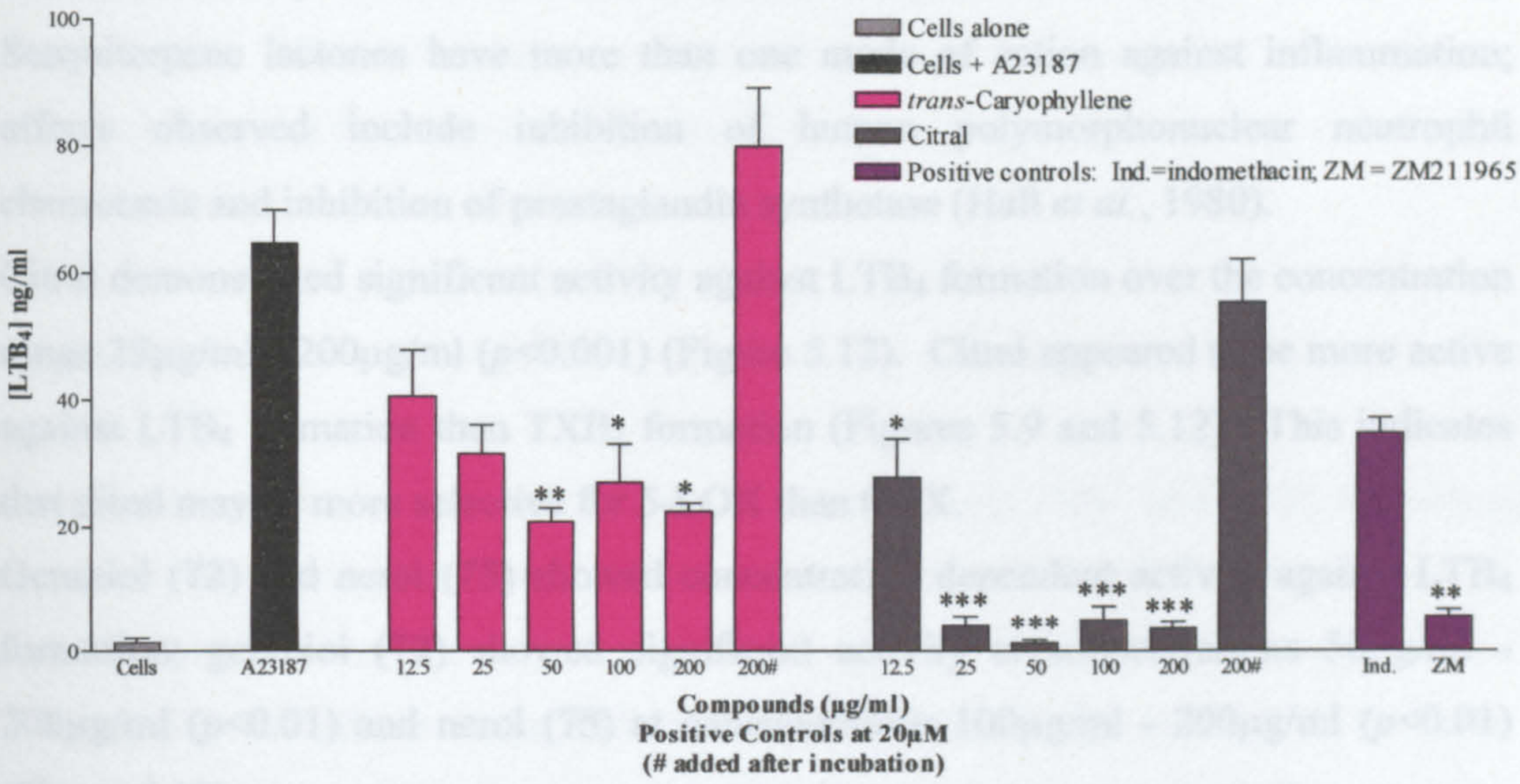


Figure 5.12. Effect of the pure compounds *trans*-caryophyllene and citral, and two reference compounds on LTB_4 generation in rat peritoneal leukocytes (n=3, except cells alone: n=6 and A23187 controls: n=24).

* $p<0.05$; ** $p<0.01$; *** $p<0.001$

The absence of activity against TXB_2 formation (refer to 5.2.3.1, Figure 5.9) suggests *trans*-caryophyllene (**82**) is not an inhibitor of COX, but may be an inhibitor of 5-LOX. This terpenoid has also been detected in *Centella asiatica* (Asakawa *et al.*, 1982), and may have influenced eicosanoid generation in the assays investigating this

plant (refer to 5.2.1, Figures 5.2 and 5.3). *Trans*-caryophyllene (**82**) has not previously been shown to demonstrate activity against eicosanoid generation. However, the oleoresin from *Copaifera* spp. (composed of sesquiterpenes including caryophyllene (**82**), α-copaene, β-elemene and humulene (**81**)) is reported to inhibit carageenan-induced oedema in rats in a dose dependent manner (Handa *et al.*, 1992). These sesquiterpenes were also found to be present in the active *M. officinalis* essential oil (refer to Chapter 2, 2.2.7.1), and so may have contributed to the effects against eicosanoid generation observed here.

Other sesquiterpenoid compounds are reported to be anti-inflammatory, including (-)-α-bisabolol, γ-cadinene, caryophyllene oxide (**78**), chamazulene, cuparene and guaizulene (Bingöl and Şener, 1995; Handa *et al.*, 1992; Harborne and Baxter, 1993). The sesquiterpene lactone hydroxyachillin isolated from *Tanacetum microphyllum* is anti-inflammatory *in vivo* and *in vitro*, and inhibits 5-LOX (Abad *et al.*, 1994). Sesquiterpene lactones have more than one mode of action against inflammation; effects observed include inhibition of human polymorphonuclear neutrophil chemotaxis and inhibition of prostaglandin synthetase (Hall *et al.*, 1980).

Citral demonstrated significant activity against LTB_4 formation over the concentration range 25μg/ml - 200μg/ml ($p<0.001$) (Figure 5.12). Citral appeared to be more active against LTB_4 formation than TXB_2 formation (Figures 5.9 and 5.12). This indicates that citral may be more selective for 5-LOX than COX.

Geraniol (**72**) and nerol (**75**) showed concentration dependent activity against LTB_4 formation; geraniol (**72**) showed significant activity at concentrations 50μg/ml - 200μg/ml ($p<0.01$) and nerol (**75**) at concentrations 100μg/ml - 200μg/ml ($p<0.01$) (Figure 5.13).

Both geraniol (**72**) and nerol (**75**) demonstrated greater activity against TXB_2 formation than against LTB_4 formation (Figures 5.10 and 5.13). This suggests that geraniol (**72**) and nerol (**75**) are more selective for COX than 5-LOX. Hydroxylation of flavonoid compounds has been suggested as an important feature for 5-LOX inhibition (Welton *et al.*, 1986). However, of the monoterpenes tested for activity against 5-LOX, it is the aldehyde citral (geranial (**70**) and neral (**71**)) which was a more potent inhibitor than the alcohols, geraniol (**72**) and nerol (**75**). This suggests an aldehyde functional group favours 5-LOX inhibition, however further investigations using other monoterpenes with alcohol and aldehyde (and perhaps other) functional groups may yield more information regarding structure-activity relationships.

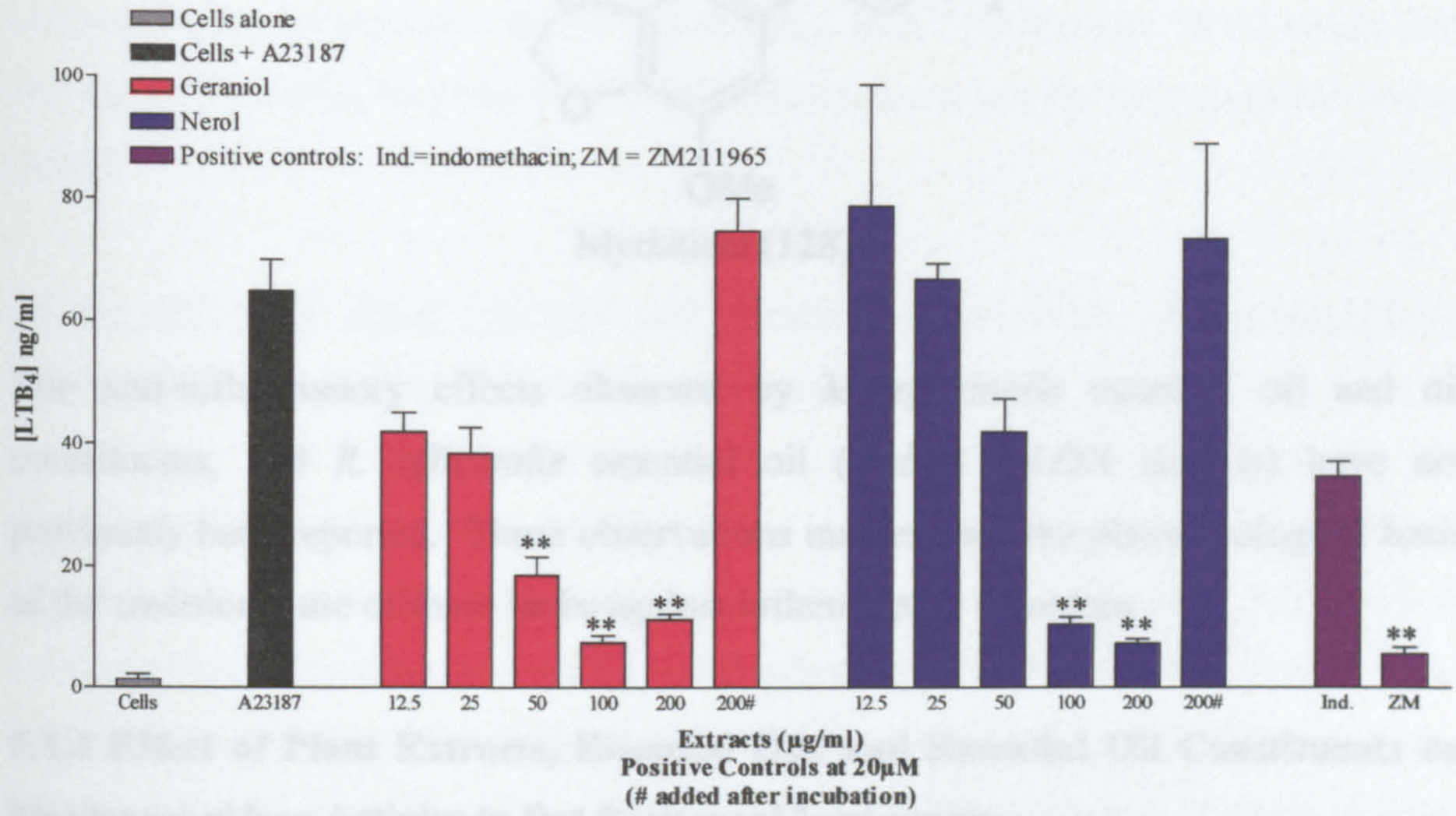
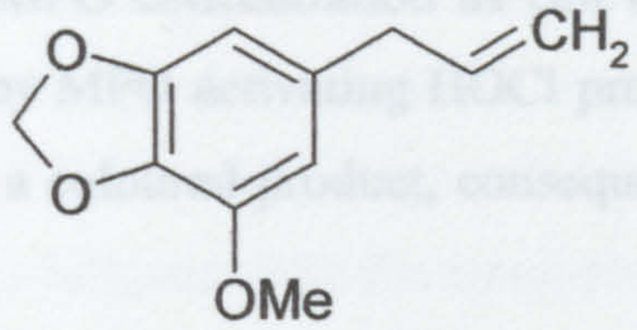


Figure 5.13. Effect of the pure compounds geraniol and nerol, and two reference compounds on LTB_4 generation in rat peritoneal leukocytes (n=3, except cells alone: n=6 and A23187 controls: n=24).

* $p<0.05$; ** $p<0.01$; *** $p<0.001$

Those test substances that exhibit activity against both TXB_2 and LTB_4 formation may not exert their effects by inhibition of the COX and 5-LOX enzymes only. It may be possible that these test substances can also influence other steps in the arachidonate cascade, such as inhibition of phospholipase A_2 activity. It has been previously reported that some eugenol (**84**), thymol and carvacrol (**147**) containing essential oils are inhibitors of prostaglandin synthesis, as are the pure compounds eugenyl acetate, capsaicin, curcumin, carvacrol (**147**) and urushiol (Wagner *et al.*, 1986). The phenylpropanoid eugenol (**84**) has also been shown to inhibit LTB_4 and HETE formation in human polymorphonuclear leukocytes and reduced carrageenin-induced foot inflammation in rats (Bennett *et al.*, 1988). Other phenylpropanoid compounds are reported to be anti-inflammatory by inhibition of prostaglandin synthesis, including myristicin (**128**) from *Myristica fragrans* (Bingöl and Şener, 1995). A chloroform extract from *Myristica fragrans*, which may contain the phenylpropanoid constituents, is also reported to have anti-inflammatory activity *in vivo* (Olajide *et al.*, 1999a).



Myristicin (**128**)

The anti-inflammatory effects observed by *M. officinalis* essential oil and oil constituents, and *R. officinalis* essential oil (against 5-LOX activity) have not previously been reported. These observations may explain the pharmacological basis of the traditional use of these herbs against inflammatory disorders.

5.2.4 Effect of Plant Extracts, Essential Oils and Essential Oil Constituents on Myeloperoxidase Activity in Rat Peritoneal Leukocytes

5.2.4.1 Myeloperoxidase Activity in Rat Peritoneal Leukocytes

An initial investigation was conducted to determine MPO catalytic activity in rat peritoneal leukocytes over time, as a function of cell number. Results show that as cell number increased, MPO activity also increased (Figure 5.14).

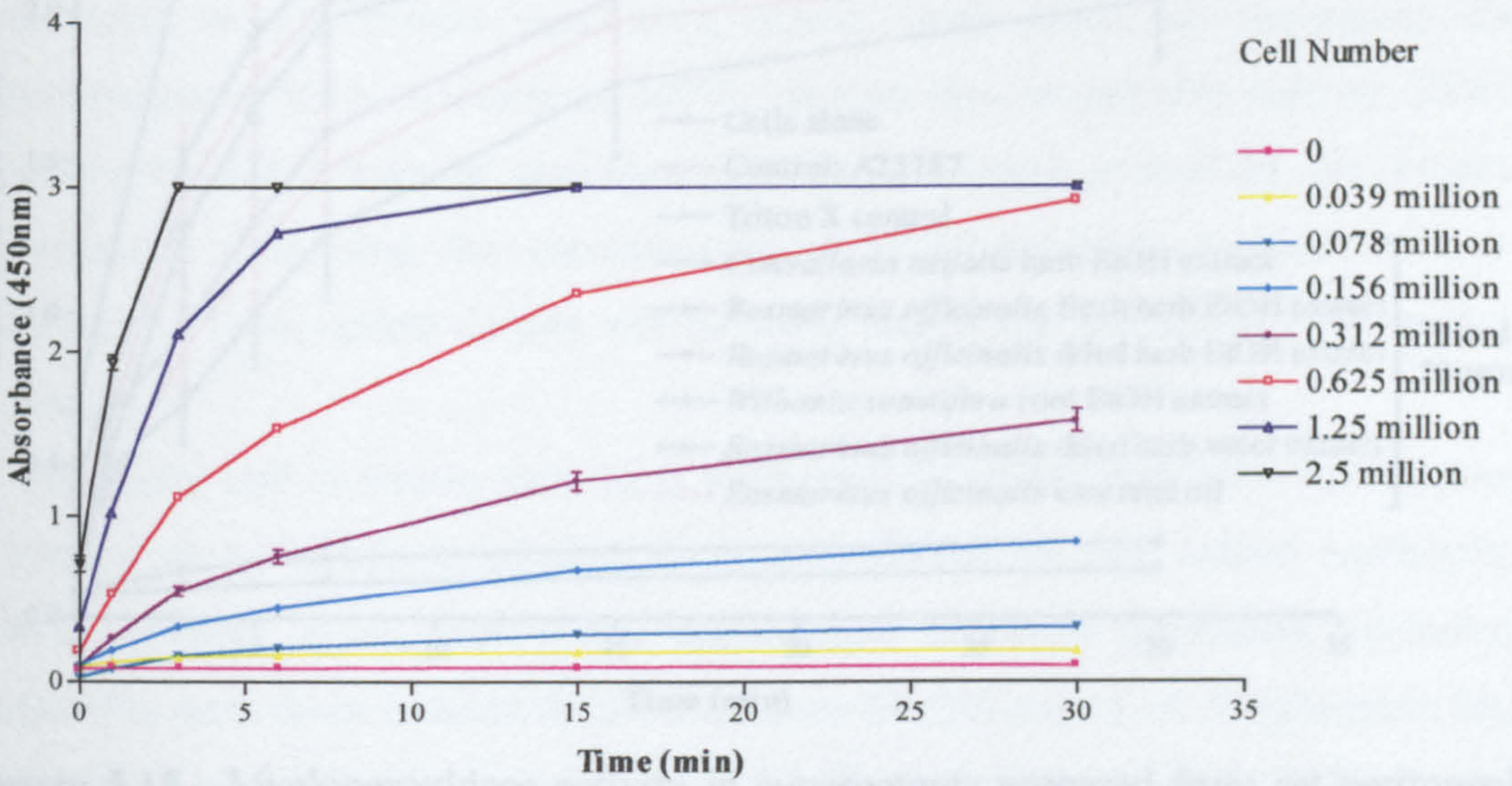


Figure 5.14. Myeloperoxidase activity in supernatants prepared from rat peritoneal leukocytes, as a function of cell number.

This reflects the increase in MPO concentration as cell number is increased. This occurrence can be explained by MPO activating HOCl production; HOCl reacts with the dye *o*-dianisidine to yield a coloured product, consequently increasing the optical density.

5.2.4.2 Effect of Plant Extracts and *Rosmarinus officinalis* Essential Oil on Myeloperoxidase Activity in Rat Peritoneal Leukocytes

Plant extracts, which produced inhibitory activity against leukocyte eicosanoid generation in previous investigations (refer to 5.2.1), were assessed for their effect on MPO activity, to investigate if the apparent effects against eicosanoid generation were actually due inhibition of enzymes in the arachidonate cascade, or due to cytotoxicity. The essential oil from *Rosmarinus officinalis* was also investigated. Results indicate that some of the extracts were cytotoxic (Figure 5.15) as MPO activity was greater in the presence of these extracts than the MPO activity in the control (A23187 alone: C2, C4, C5); however none of the test substances were as cytotoxic as the triton-X control (C10).

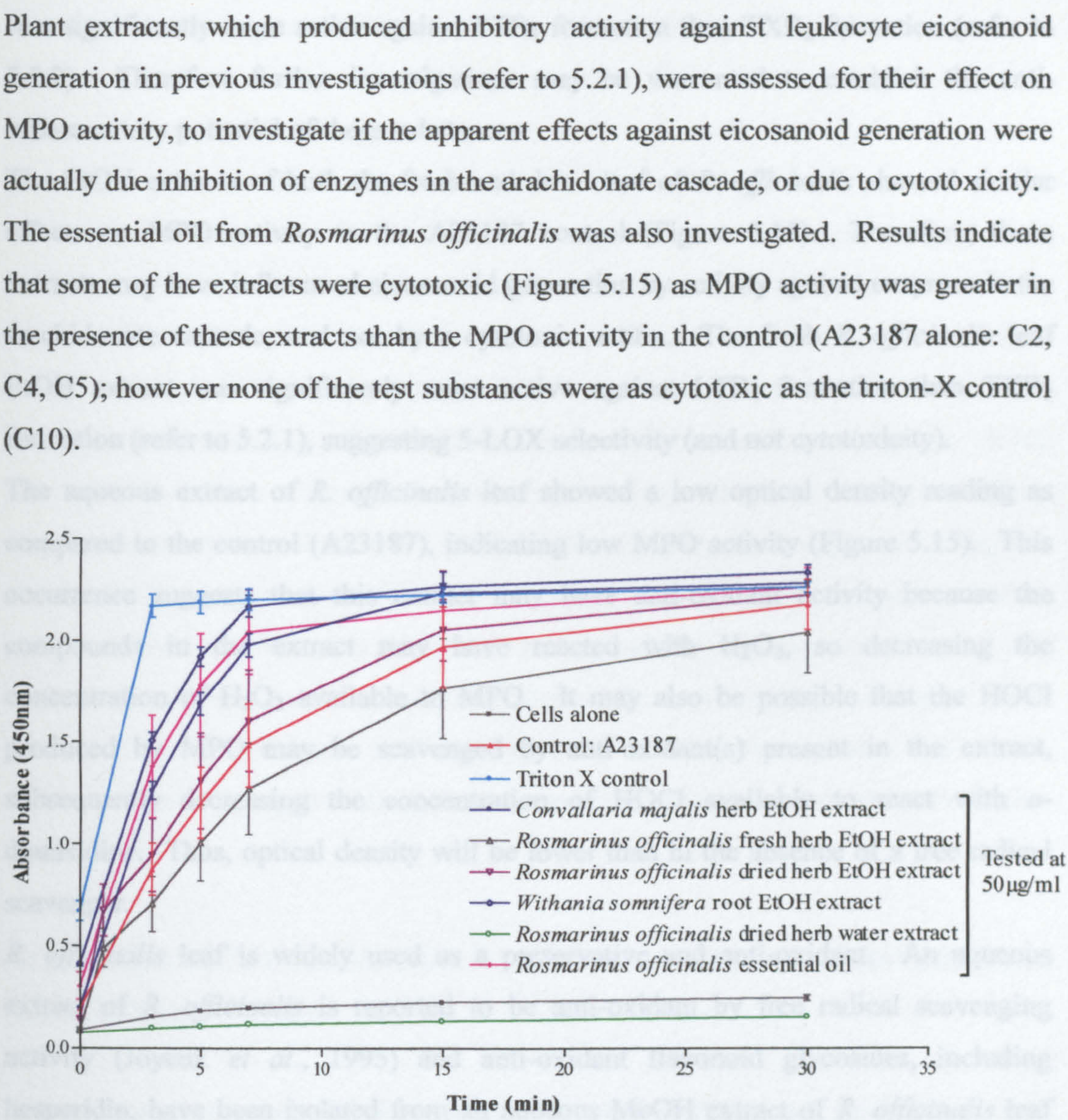


Figure 5.15. Myeloperoxidase activity in supernatants prepared from rat peritoneal leukocytes under control and A23187 stimulated conditions, in the presence of some plant extracts and *Rosmarinus officinalis* essential oil.

Of those extracts tested, the EtOH extract of *Withania somnifera* was most cytotoxic (Figure 5.15). The EtOH extract of *Convallaria majalis* and the *R. officinalis* essential oil also demonstrated cytotoxicity (Figure 5.15). Therefore the possibility that eicosanoid generation was influenced by the cytotoxicity of these test substances cannot be excluded. However, the EtOH extracts of *C. majalis* and *W. somnifera* were more active against TXB_2 than LTB_4 formation (refer to 5.2.1), which suggests compound(s) in these extracts may be more selective for COX than 5-LOX. Similarly, *R. officinalis* essential oil appeared more selective for 5-LOX, as the oil was significantly more active against LTB_4 formation than TXB_2 formation (refer to 5.2.2). Therefore further investigations may be warranted to establish the anti-inflammatory potential of these substances.

The EtOH extracts of both the fresh and dried leaf of *R. officinalis* showed similar effects on MPO activity to the A23187 control (Figure 5.15). Therefore, these extracts may have influenced eicosanoid generation by activity against enzymes in the arachidonate cascade, and not by a cytotoxic action. The fresh *R. officinalis* leaf EtOH extract was significantly more active against LTB_4 formation than TXB_2 formation (refer to 5.2.1), suggesting 5-LOX selectivity (and not cytotoxicity).

The aqueous extract of *R. officinalis* leaf showed a low optical density reading as compared to the control (A23187), indicating low MPO activity (Figure 5.15). This occurrence suggests that this extract may have anti-oxidant activity because the compounds in the extract may have reacted with H_2O_2, so decreasing the concentration of H_2O_2 available to MPO. It may also be possible that the HOCl produced by MPO may be scavenged by anti-oxidant(s) present in the extract, subsequently decreasing the concentration of HOCl available to react with *o*-dianisidine. Thus, optical density will be lower than in the absence of a free radical scavenger.

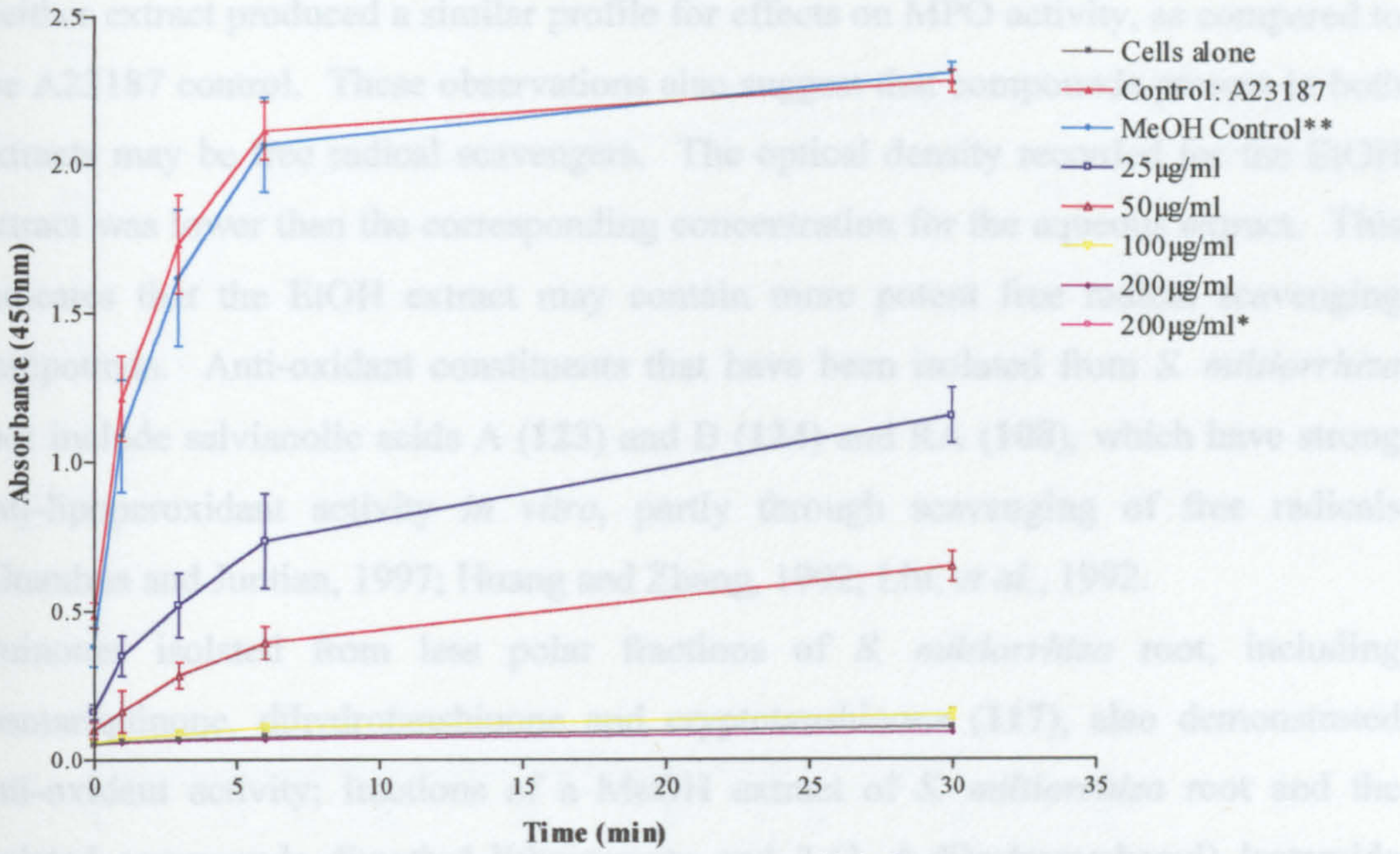
R. officinalis leaf is widely used as a preservative and anti-oxidant. An aqueous extract of *R. officinalis* is reported to be anti-oxidant by free radical scavenging activity (Joyeux *et al.*, 1995) and anti-oxidant flavonoid glycosides, including hesperidin, have been isolated from an aqueous MeOH extract of *R. officinalis* leaf (Cuvelier *et al.*, 1996; Okamura *et al.*, 1994). The anti-oxidant activity of flavonoids is well documented (Takahama, 1985; Torel *et al.*, 1986). It is therefore possible that flavonoid compounds in the aqueous extract of *R. officinalis* leaf were responsible for the low optical density readings observed. Some other phenolic compounds such as

coumarins and tannins are also known free radical scavengers (Bouchet *et al.*, 1998; Hoult and Payà, 1996; Montesinos *et al.*, 1991), and their presence may also contribute to an anti-oxidant effect.

The presence of free radical scavengers may have influenced eicosanoid generation. Both COX and 5-LOX are reported to catalyse free radical peroxidation of AA (Hanel and Lands, 1982; Riendeau *et al.*, 1989). It has been proposed that inhibition of 5-LOX is due to the reaction of the inhibitor with free radicals generated at the enzyme's active site (Takahama, 1985; Torel *et al.*, 1986); therefore in the presence of free radical scavengers, inhibition of the enzyme by this mechanism would be decreased, thus increasing eicosanoid generation. This occurrence may explain the apparent inactivity of the aqueous extract of *R. officinalis* leaf against eicosanoid generation (refer to 5.2.1, Figures 5.2 and 5.3). However, the potency of the inhibition of COX and 5-LOX by flavonoid compounds was not found to significantly correlate with anti-oxidant activity, suggesting that scavenging effects on free radicals (generated at the active site of the enzyme) do not explain enzyme inhibition (Laughton *et al.*, 1991). It is apparent that the relationship between the anti-oxidant and eicosanoid inhibiting effects of compounds requires further investigation. It has also been found that a combination of iron-chelating and iron ion-reducing effects may be necessary for selective 5-LOX inhibition by phenolic compounds (Laughton *et al.*, 1991); it may be useful to assess the effects of *R. officinalis* aqueous extract on iron chelation and reduction to further understand the apparent inactivity of this extract against eicosanoid generation. Free-radical scavenging effects of other extracts (e.g. *M. officinalis* leaf extracts; refer to 5.2.4.4) may also explain the observed inactivity against eicosanoid generation, and this also warrants further investigation.

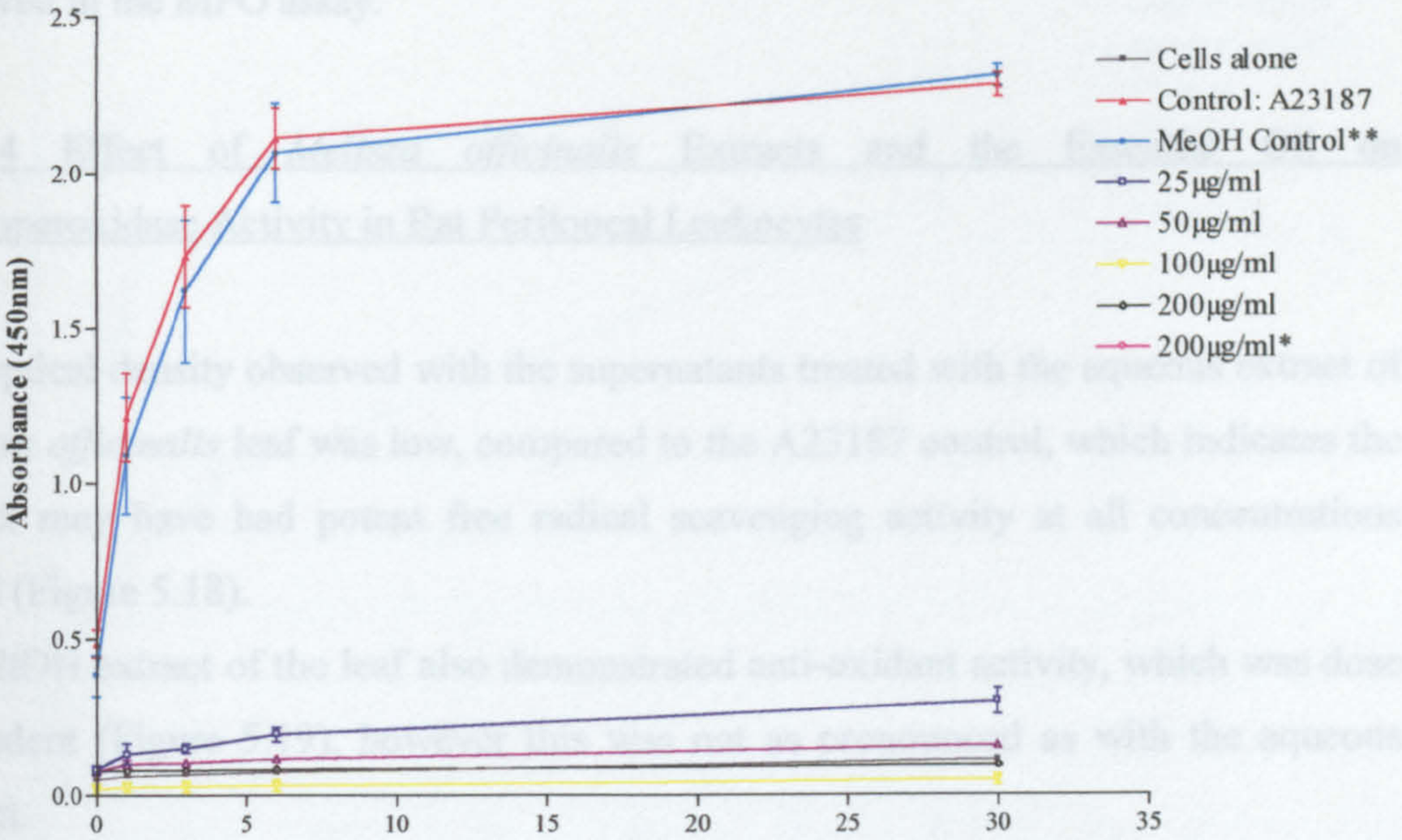
5.2.4.3 Effect of *Salvia miltiorrhiza* Root Extracts on Myeloperoxidase Activity in Rat Peritoneal Leukocytes

Both the aqueous and EtOH extracts of *S. miltiorrhiza* root produced a dose dependent decrease in optical density, as the concentration of extract was increased (Figures 5.16 and 5.17).



* Control: 200µg/ml *Salvia miltiorrhiza* root water extract added after
** MeOH added after cells

Figure 5.16. Myeloperoxidase activity in supernatants prepared from rat peritoneal leukocytes under control and A23187 stimulated conditions, in the presence of *Salvia miltiorrhiza* root aqueous extract.



* Control: 200µg/ml *Salvia miltiorrhiza* root EtOH extract added after
** MeOH added after cells

Figure 5.17. Myeloperoxidase activity in supernatants prepared from rat peritoneal leukocytes under control and A23187 stimulated conditions, in the presence of *Salvia miltiorrhiza* root ethanolic extract.

Neither extract produced a similar profile for effects on MPO activity, as compared to the A23187 control. These observations also suggest that compounds present in both extracts may be free radical scavengers. The optical density recorded for the EtOH extract was lower than the corresponding concentration for the aqueous extract. This indicates that the EtOH extract may contain more potent free radical scavenging compounds. Anti-oxidant constituents that have been isolated from *S. miltiorrhiza* root include salvianolic acids A (**123**) and B (**124**) and RA (**108**), which have strong anti-lipoperoxidant activity *in vitro*, partly through scavenging of free radicals (Guanhua and Juntian, 1997; Huang and Zhang, 1992; Liu, *et al.*, 1992.

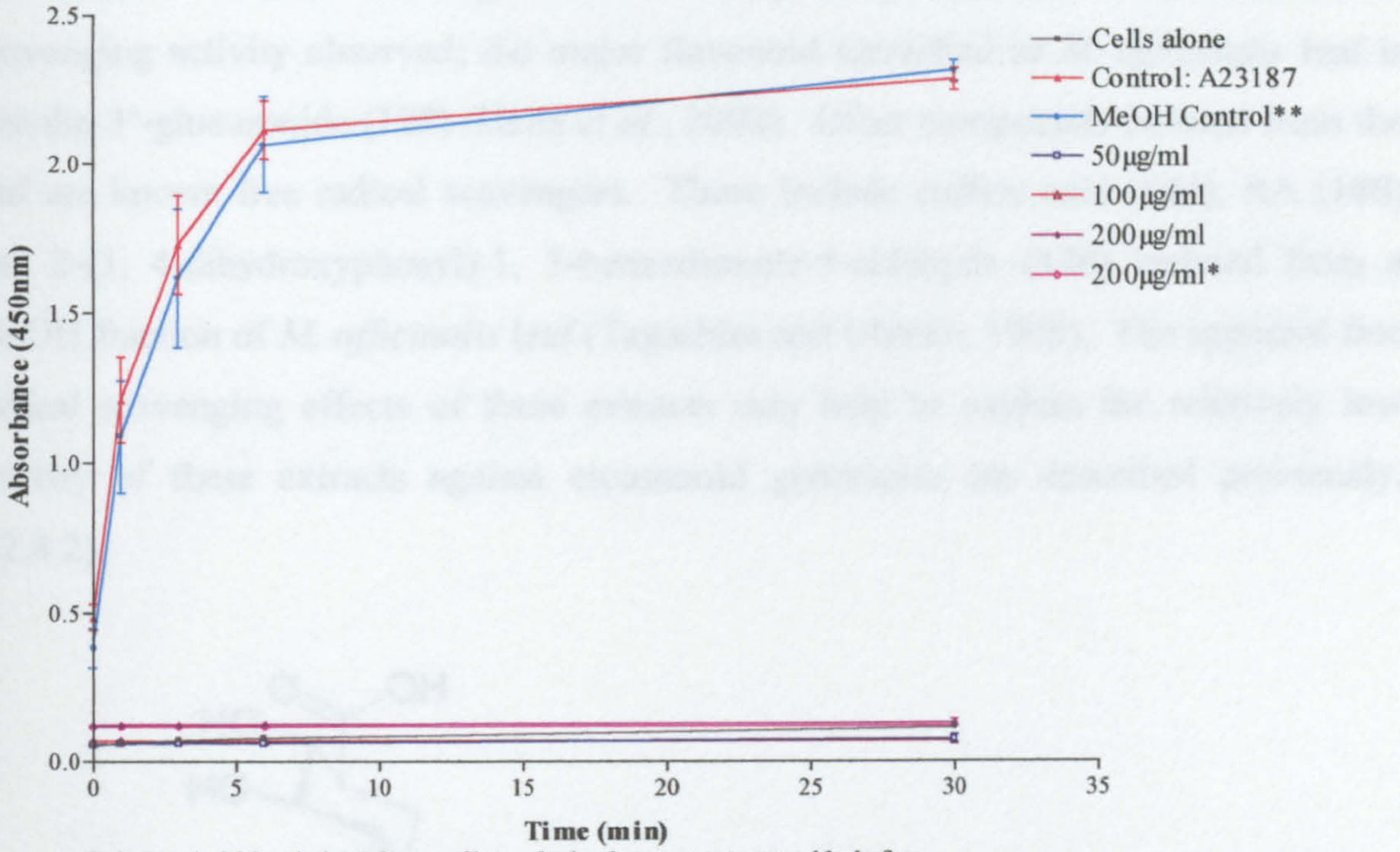
Quinones isolated from less polar fractions of *S. miltiorrhiza* root, including rosmariquinone, dihydrotanshinone and cryptotanshinone (**117**), also demonstrated anti-oxidant activity; fractions of a MeOH extract of *S. miltiorrhiza* root and the isolated compounds dimethyl lithspermate and 3-(3, 4-dihydroxyphenyl) lactamide (**140**) were identified as free radical scavengers (Kang *et al.*, 1997; Weng and Gordon, 1992; Zhao *et al.*, 1996; also refer to Chapter 6, 6.1.2.2.6).

It appears that the presence of these free radical scavenging compounds in the *S. miltiorrhiza* extracts, may be responsible for the possible anti-oxidant effects observed in the MPO assay.

5.2.4.4 Effect of *Melissa officinalis* Extracts and the Essential Oil on Myeloperoxidase Activity in Rat Peritoneal Leukocytes

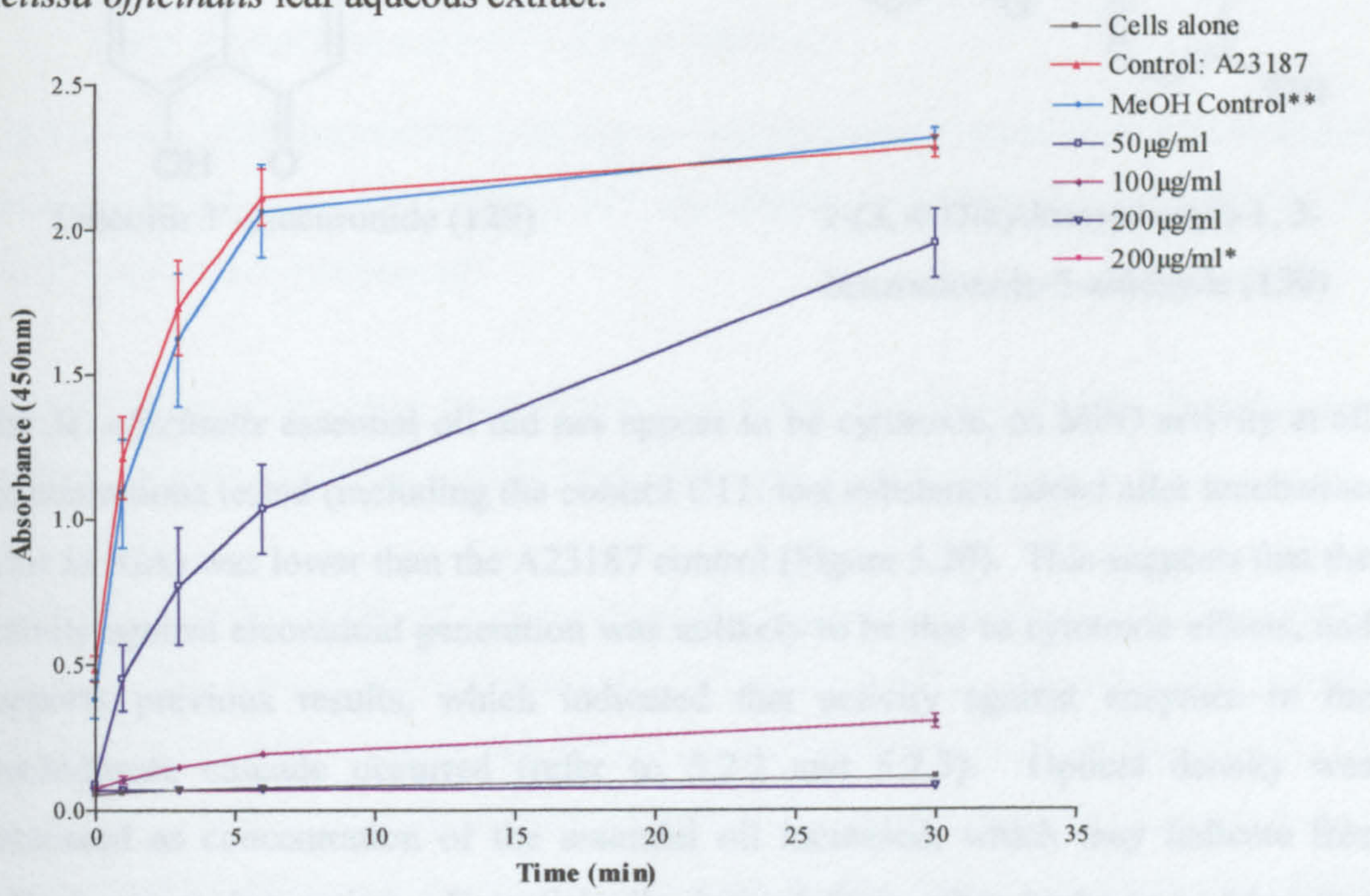
The optical density observed with the supernatants treated with the aqueous extract of *Melissa officinalis* leaf was low, compared to the A23187 control, which indicates the extract may have had potent free radical scavenging activity at all concentrations tested (Figure 5.18).

The EtOH extract of the leaf also demonstrated anti-oxidant activity, which was dose dependent (Figure 5.19), however this was not as pronounced as with the aqueous extract.



* Control: 200µg/ml *Melissa officinalis* herb water extract added after
** MeOH added after cells

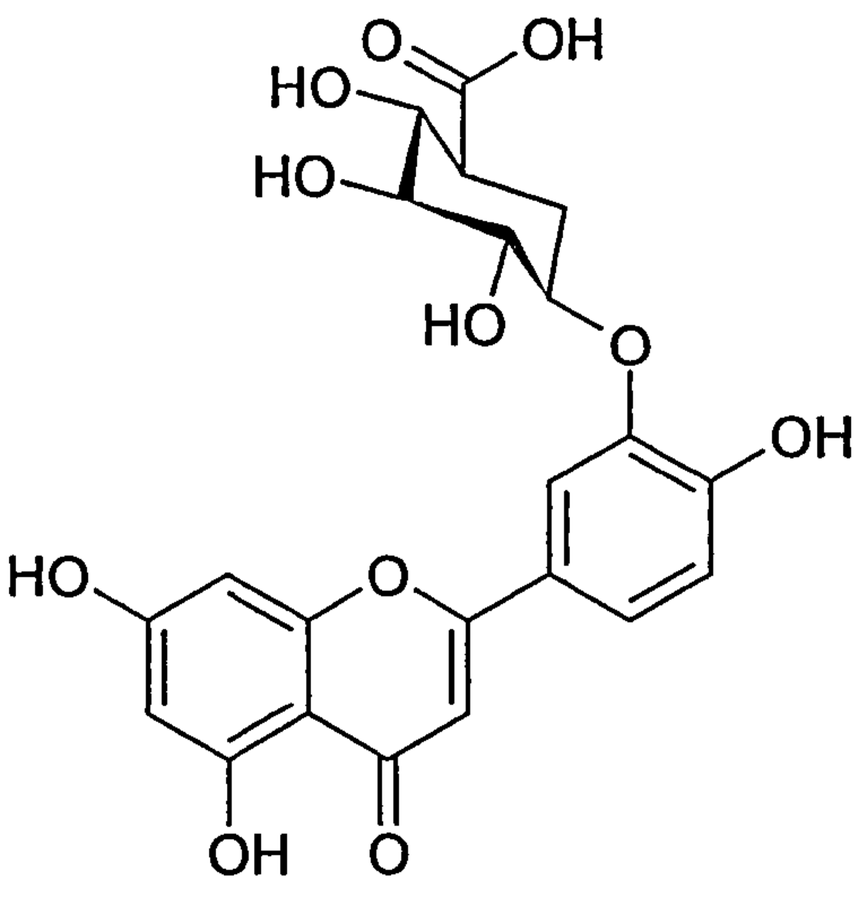
Figure 5.18. Myeloperoxidase activity in supernatants prepared from rat peritoneal leukocytes under control and A23187 stimulated conditions, in the presence of *Melissa officinalis* leaf aqueous extract.



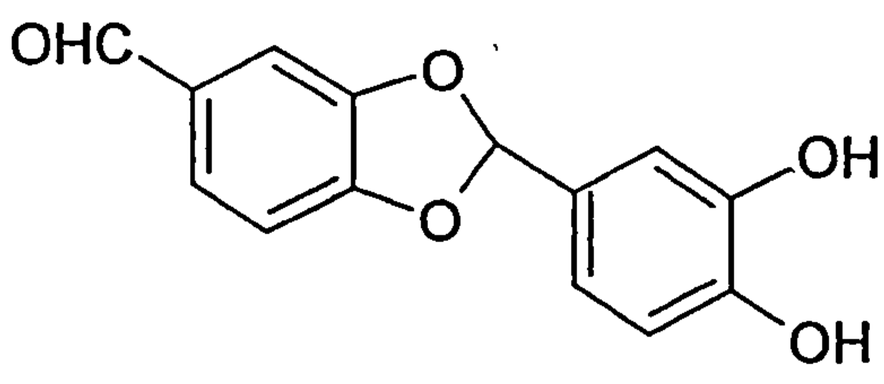
* Control: 200µg/ml *Melissa officinalis* herb EtOH extract added after
** MeOH added after cells

Figure 5.19. Myeloperoxidase activity in supernatants prepared from rat peritoneal leukocytes under control and A23187 stimulated conditions, in the presence of *Melissa officinalis* leaf ethanolic extract.

The flavonoid content of *M. officinalis* leaf may have contributed to the free-radical scavenging activity observed; the major flavonoid identified in *M. officinalis* leaf is luteolin 3'-glucuronide (**129**) (Heitz *et al.*, 2000). Other compounds isolated from the leaf are known free radical scavengers. These include caffeic acid (**106**), RA (**108**) and 2-(3, 4-dihydroxyphenyl)-1, 3-benzodioxole-5-aldehyde (**130**) isolated from a MeOH fraction of *M. officinalis* leaf (Tagashira and Ohtake, 1998). The apparent free radical scavenging effects of these extracts may help to explain the relatively low activity of these extracts against eicosanoid generation (as described previously, 5.2.4.2).



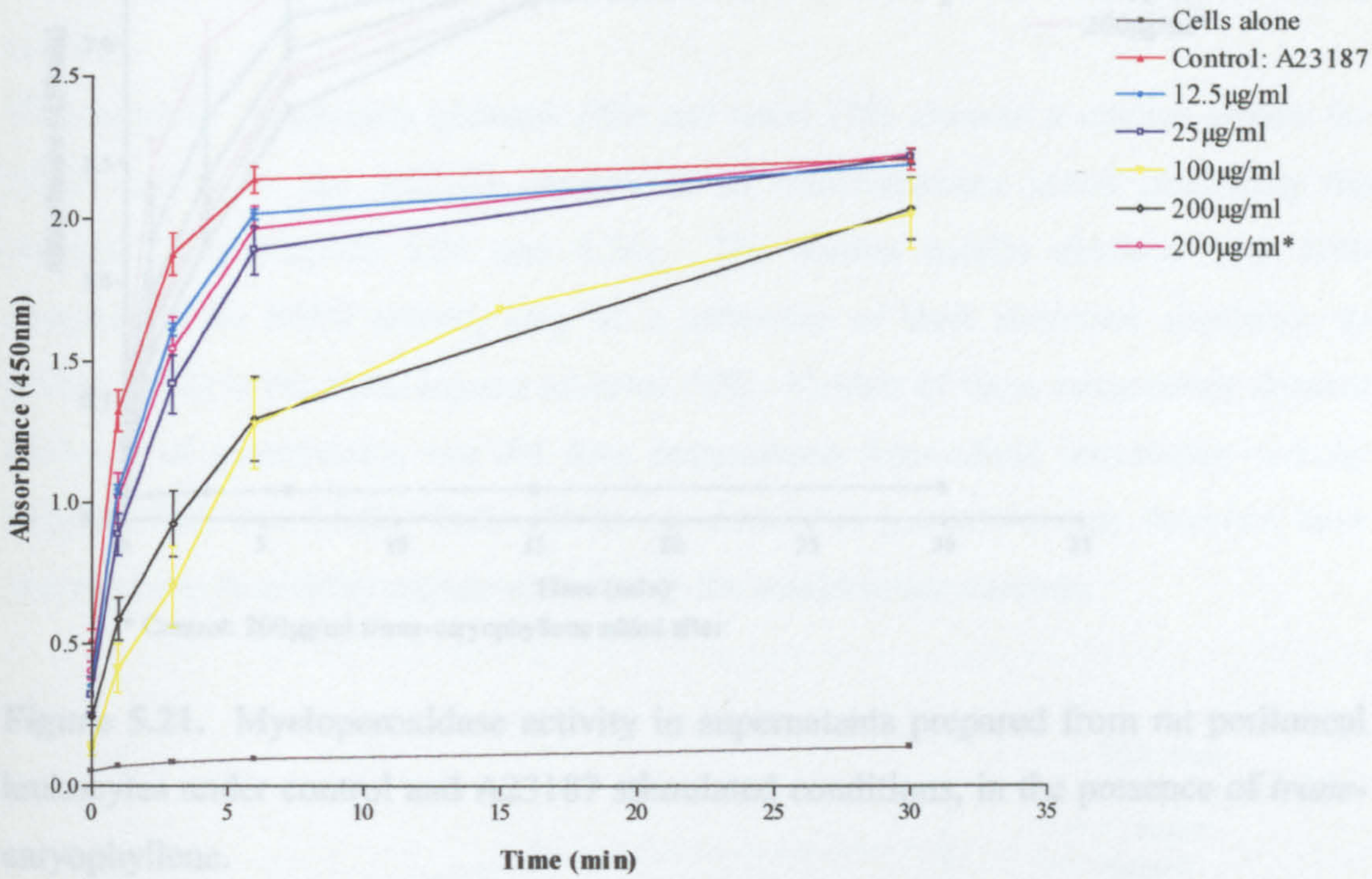
Luteolin 3'-glucuronide (**129**)



2-(3, 4-Dihydroxyphenyl)-1, 3-benzodioxole-5-aldehyde (**130**)

The *M. officinalis* essential oil did not appear to be cytotoxic, as MPO activity at all concentrations tested (including the control C11: test substance added after incubation prior to RIA) was lower than the A23187 control (Figure 5.20). This suggests that the activity against eicosanoid generation was unlikely to be due to cytotoxic effects, and supports previous results, which indicated that activity against enzymes in the arachidonate cascade occurred (refer to 5.2.2 and 5.2.3). Optical density was decreased as concentration of the essential oil increased, which may indicate free radical scavenging action. Essential oils derived from other herbs (e.g. *Myristica fragrans*, *Thymus vulgaris*) have been identified as being anti-oxidant (Dorman *et al.*, 1995). The monoterpenes carvacrol (**147**) and thymol (although not identified in the *M. officinalis* essential oil) are also anti-oxidant, and are effective scavengers of

peroxyl radicals (Aesbach *et al.*, 1994). It is therefore feasible that the apparent free radical scavenging activity of *M. officinalis* essential oil may be due to the monoterpene content, perhaps citral (refer to 5.2.4.5).



* Control: 200µg/ml *Melissa officinalis* essential oil added after

Figure 5.20. Myeloperoxidase activity in supernatants prepared from rat peritoneal leukocytes under control and A23187 stimulated conditions, in the presence of *Melissa officinalis* essential oil.

5.2.4.5 Effect of the Pure Compounds *Trans*-Caryophyllene, Citral, Geraniol and Nerol on Myeloperoxidase Activity in Rat Peritoneal Leukocytes

Trans-caryophyllene (**82**) did not demonstrate cytotoxicity or free radical scavenging activity; each concentration tested (and the control, C11) showed MPO activity similar to the A23187 control, but did not exceed that of the control (Figure 5.21). It is therefore possible that the activity against LTB_4 generation (refer to 5.2.3.2) was due to 5-LOX inhibition and not due to cytotoxicity (the lack of activity against TXB_2 generation (refer to 5.2.3.1) is further evidence for this).

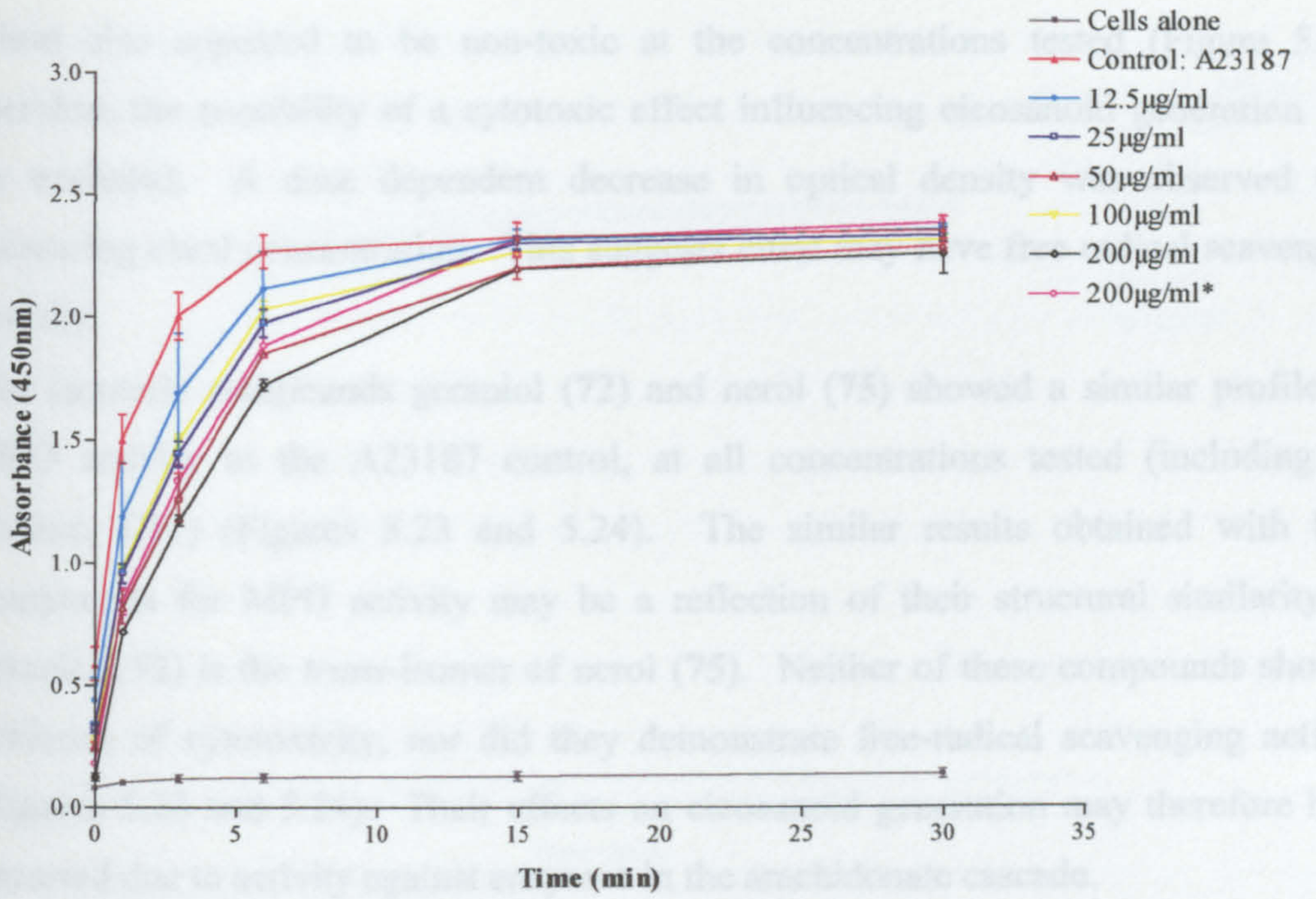


Figure 5.21. Myeloperoxidase activity in supernatants prepared from rat peritoneal leukocytes under control and A23187 stimulated conditions, in the presence of *trans*-caryophyllene.

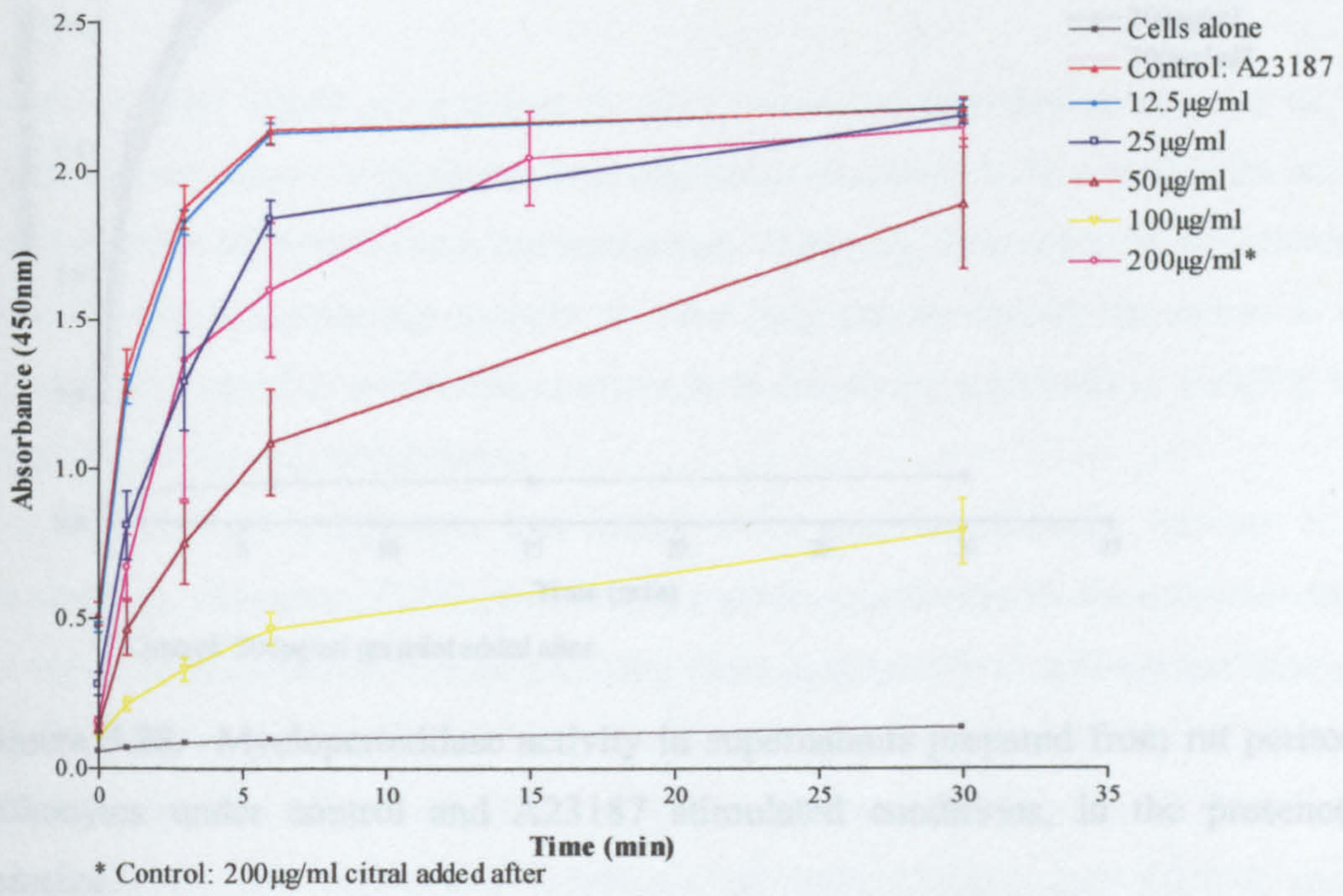
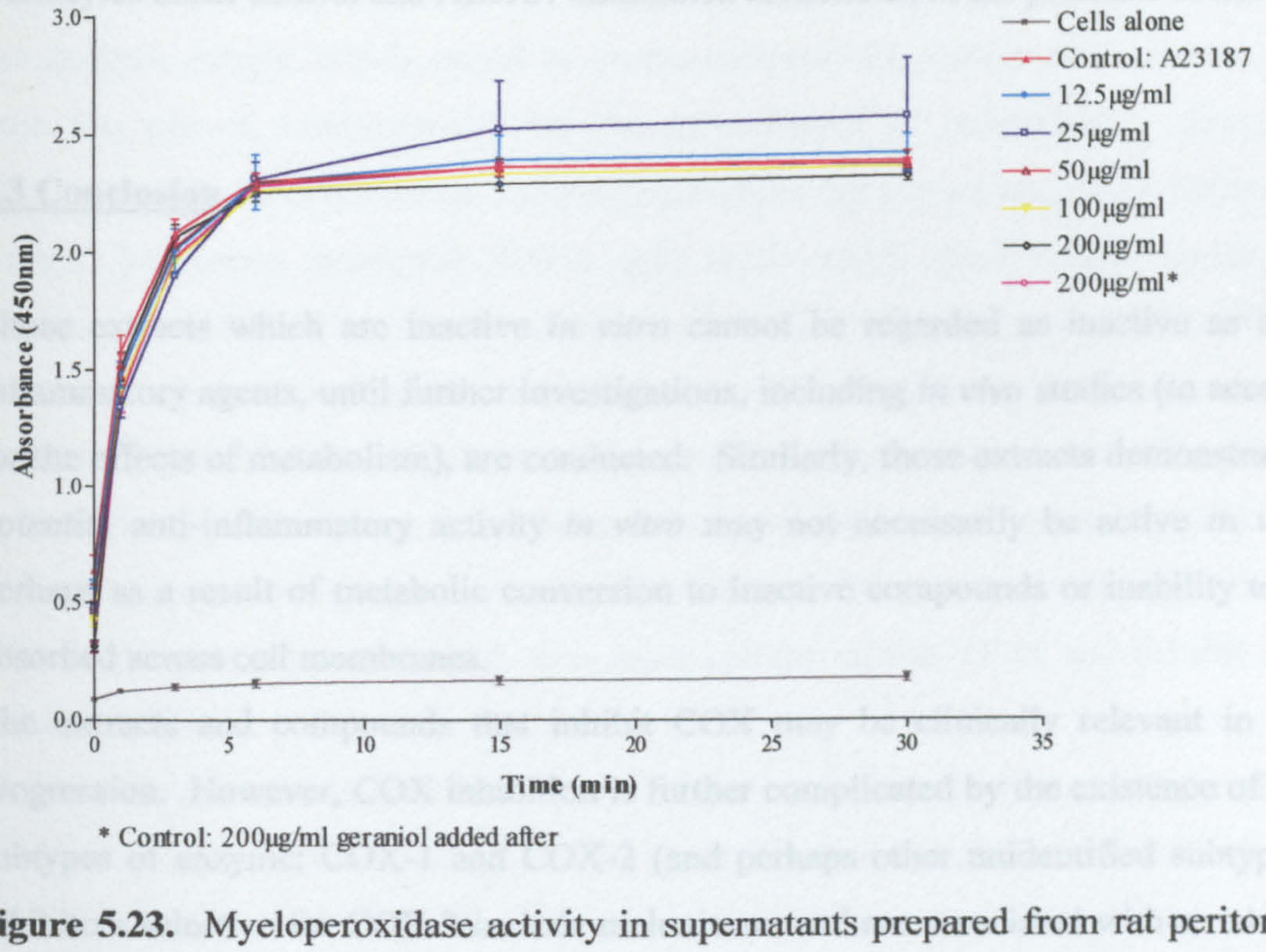


Figure 5.22. Myeloperoxidase activity in supernatants prepared from rat peritoneal leukocytes under control and A23187 stimulated conditions, in the presence of citral.

Citral also appeared to be non-toxic at the concentrations tested (Figure 5.22); therefore the possibility of a cytotoxic effect influencing eicosanoid generation may be excluded. A dose dependent decrease in optical density was observed with increasing citral concentration. This suggests citral may have free radical scavenging activity.

The isomeric compounds geraniol (**72**) and nerol (**75**) showed a similar profile for MPO activity to the A23187 control, at all concentrations tested (including the control, C11) (Figures 5.23 and 5.24). The similar results obtained with both compounds for MPO activity may be a reflection of their structural similarity, as geraniol (**72**) is the *trans*-isomer of nerol (**75**). Neither of these compounds showed evidence of cytotoxicity, nor did they demonstrate free-radical scavenging activity (Figures 5.23 and 5.24). Their effects on eicosanoid generation may therefore have occurred due to activity against enzymes in the arachidonate cascade.



* Control: 200µg/ml geraniol added after

Figure 5.23. Myeloperoxidase activity in supernatants prepared from rat peritoneal leukocytes under control and A23187 stimulated conditions, in the presence of geraniol.

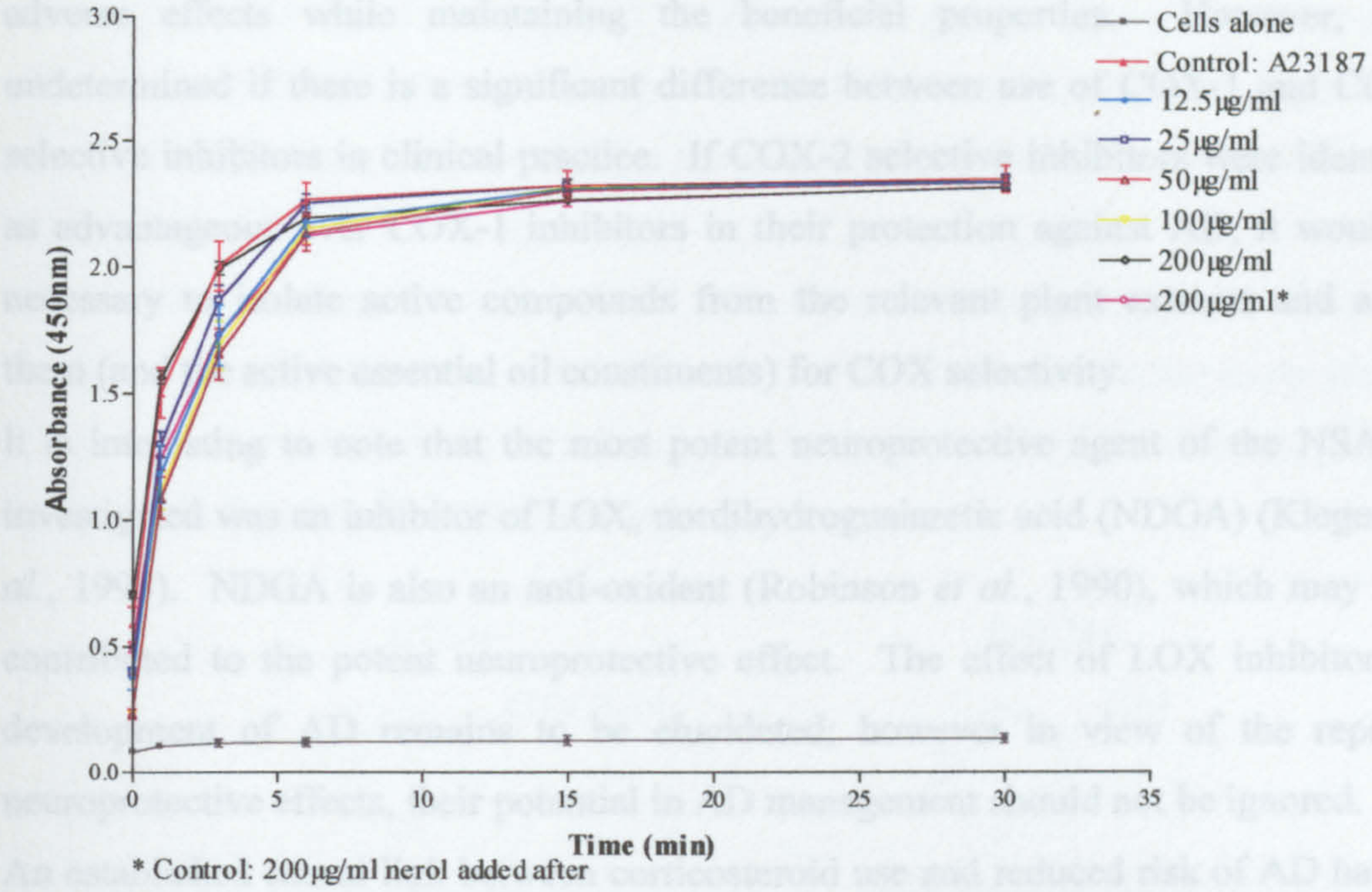


Figure 5.24. Myeloperoxidase activity in supernatants prepared from rat peritoneal leukocytes under control and A23187 stimulated conditions, in the presence of nerol.

5.3 Conclusion

Those extracts which are inactive *in vitro* cannot be regarded as inactive as anti-inflammatory agents, until further investigations, including *in vivo* studies (to account for the effects of metabolism), are conducted. Similarly, those extracts demonstrating potential anti-inflammatory activity *in vitro* may not necessarily be active *in vivo*, perhaps as a result of metabolic conversion to inactive compounds or inability to be absorbed across cell membranes.

The extracts and compounds that inhibit COX may be clinically relevant in AD progression. However, COX inhibition is further complicated by the existence of two subtypes of enzyme: COX-1 and COX-2 (and perhaps other unidentified subtypes). Inhibitors selective for COX-2 include meloxicam, and are associated with a reduced incidence of adverse effects (Furst, 1997); inhibitors selective for COX-1 include indomethacin, and equipotent inhibitors of both COX-1 and COX-2 include diclofenac. The neuroprotective potency of NSAIDs is not related to their selectivity for COX-1 or COX-2 (Klegeris *et al.*, 1999). This is of therapeutic importance as inhibitors selective for COX-2, or other unidentified COX subtypes would minimise

adverse effects while maintaining the beneficial properties. However, it is undetermined if there is a significant difference between use of COX-1 and COX-2 selective inhibitors in clinical practice. If COX-2 selective inhibitors were identified as advantageous over COX-1 inhibitors in their protection against AD, it would be necessary to isolate active compounds from the relevant plant extracts and assess them (and the active essential oil constituents) for COX selectivity.

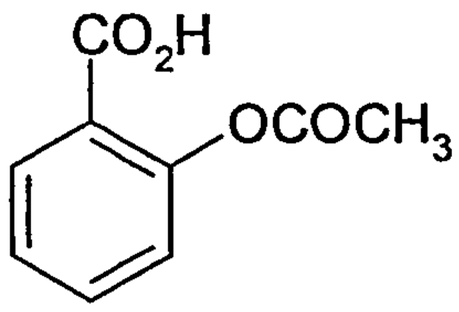
It is interesting to note that the most potent neuroprotective agent of the NSAID's investigated was an inhibitor of LOX, nordihydroguaiaretic acid (NDGA) (Klegeris *et al.*, 1999). NDGA is also an anti-oxidant (Robinson *et al.*, 1990), which may have contributed to the potent neuroprotective effect. The effect of LOX inhibitors on development of AD remains to be elucidated; however in view of the reported neuroprotective effects, their potential in AD management should not be ignored.

An established causal link between corticosteroid use and reduced risk of AD has not been established, however some studies report that corticosteroids effectively suppress complement activation in AD (Aisen, 1996). Adverse effects that include psychogenic effects, which would be contra-indicated in Alzheimer's patients, also limit the use of corticosteroids for the management of inflammatory disorders. Corticosteroids (dexamethasone and prednisone) did not protect against neurotoxicity induced by human monocytic THP-1 cells (cells which show similar toxicity to human microglia), unlike the neuroprotective NSAIDs (Klegeris *et al.*, 1999). Those plant extracts that may possess a steroid-like anti-inflammatory action are also of therapeutic potential. Steroidal compounds, such as those present in *Centella asiatica* and *Withania somnifera*, may demonstrate a protective action against the inflammatory processes that occur in AD, but lack the potentially serious adverse effects. Such compounds which also show activity against COX and 5-LOX may enhance efficacy.

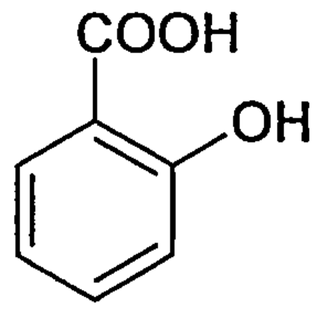
Finally, it is essential to consider that activity *in vitro* may not always reflect activity *in vivo*. The pharmacokinetics (including the absorption, metabolism, transfer across the BBB and rate of elimination) *in vivo* may influence the clinical effects. For example, citral and geraniol (**72**) are reported to be metabolised to hildebrandt acid and dihydrohildebrandt acid by the liver of mammals (Asano and Yamakawa, 1950; Chadha and Madyastha, 1984; Ishida *et al.*, 1989). Therefore the pharmacology and pharmacokinetics of any metabolites also require investigation. Toxicity *in vivo* is also an important consideration. For example, some essential oil constituents may be

toxic (Tisserand and Balacs, 1996) at therapeutic doses. Essential oils may be administered via inhalation, as therapeutic effects have been observed, and essential oil constituents have been detected in plasma, following this route of administration, (Jäger *et al.*, 1992; Kovar *et al.*, 1987).

Anti-inflammatory compounds isolated from these plants may not only be new drugs themselves, but may also act as templates for the development of other more effective drugs. For example, aspirin (acetyl salicylic acid) (**131**) is derived from the phenolic compound salicylic acid (**132**) from *Salix* spp.



Aspirin (**131**)



Salicylic acid (**132**)

Other phenolic compounds with related chemical structures, such as caffeic acid (**106**) and RA (**108**), may act as templates for drug synthesis.

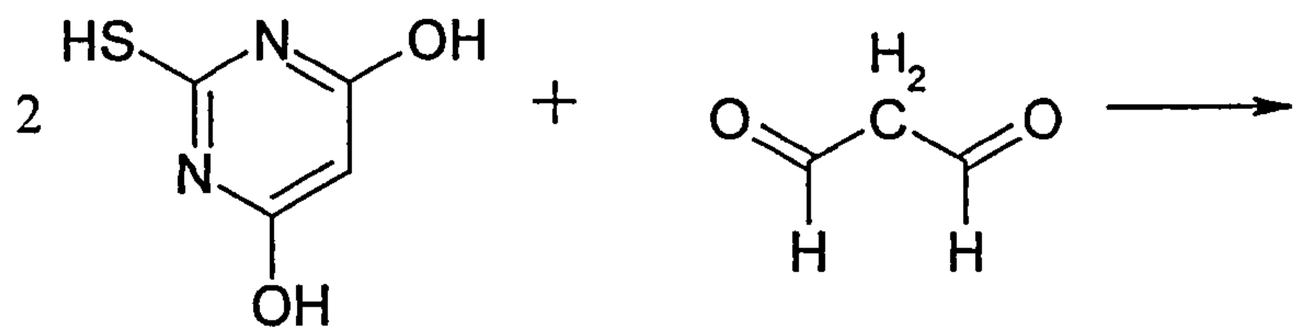
From the analysis of the results from these investigations and the vast array of phytochemicals reported to be anti-inflammatory, there is potential for those plant extracts that were effective against eicosanoid generation to yield new therapeutic agents for the management of inflammatory disorders such as AD.

CHAPTER 6

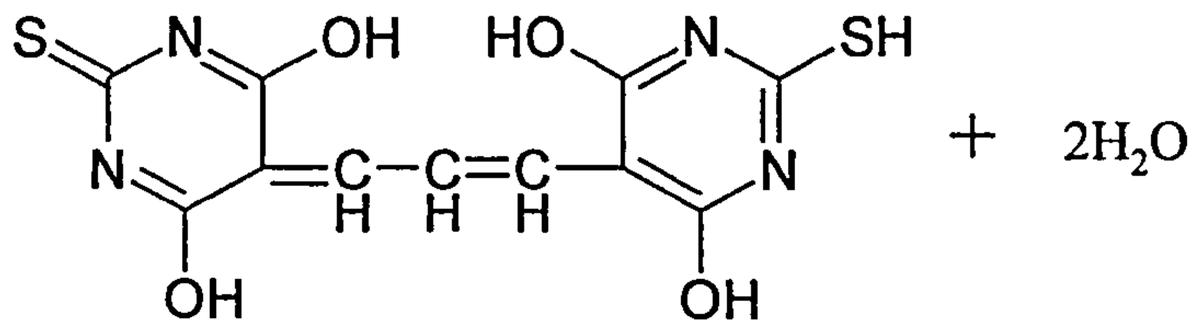
Effect of Plant Extracts and Essential Oils in Other Bioassay Investigations

6.1 Assessment of Anti-Oxidant Activity of Plant Extracts and Essential Oils Using a Phospholipid Peroxidation Assay

Most lipid peroxidation *in vivo* is metal-ion dependent (Halliwell and Gutteridge, 1995). Lipid peroxide arising from lipid peroxidation in cells is cytotoxic, causing disruption of cell membranes (Ando *et al.*, 1990); this mechanism has been found to be one of the major biochemical markers of aging. Therefore to identify any anti-oxidant activity of plant extracts, their ability to inhibit iron-stimulated lipid peroxidation was assessed. To assess inhibition of lipid peroxidation a thiobarbituric acid (TBA) (**133**) test was employed that was a modification of the method described by Uchiyama and Mihara (1978). The basis of this assay is that peroxidation is initiated in bovine brain liposomes by the presence of Fe^{3+} and ascorbic acid and small quantities of malonaldehyde (MDA) (**134**) are formed. MDA (**134**) reacts in the TBA (**133**) test to generate the coloured $(TBA)_2$-MDA adduct, perhaps by the following reaction, that absorbs light at 532nm in acidic solution, and is readily extractable into organic solvents.



TBA (**133**) MDA (**134**)



6.1.1 Method

6.1.1.1 Materials

Ascorbic acid, butylated hydroxytoluene (BHT), bovine brain extract (Type VII), *n*-butanol, $FeCl_3$, glass balls (2mm diameter), propyl gallate (PpG), phosphate buffered saline (PBS) tablets (pH 7.4) and thiobarbituric acid (TBA) were purchased from Sigma, Fancy Road, Poole, Dorset, England. Plant extracts and essential oils were obtained as described previously; *Melissa officinalis* essential oil was obtained from Fragrant Earth, Taunton, England (refer to Chapter 2, 2.1.1.1).

6.1.1.2 Preparation of Assay Solutions

Preparation of Liposomes

50mg bovine brain extract and 10ml PBS (10mM) was sonicated in the presence of 7 glass balls. Sonication was conducted in an ice water bath (to minimize peroxidation of liposomes) until a homogenous suspension was obtained.

Preparation of Other Assay Solutions

Assay solutions were prepared as follows:

Chemical	Dissolution Material	Concentration
Ascorbic acid	Distilled water	1mM
BHT	EtOH	2%
$FeCl_3$	Distilled water	1mM
HCl	Distilled water	25%
TBA	50mM NaOH	1%

6.1.1.3 Assay Method

0.1ml test substance (dissolved in EtOH), 0.2ml phospholipid liposomes (5mg/ml), 0.1ml $FeCl_3$ (100μM) and 0.1ml ascorbic acid (100μM) were made up to a total volume (per tube) of 1.0ml with PBS. Controls were also made up to 1.0ml with PBS

(Tables 6.1 and 6.2). After vortex mixing, all tubes were incubated at 37°C for 1hr. During incubation, the reaction of iron complexes with lipid peroxides generates a range of products, including MDA (**2**).

Table 6.1. Controls for phospholipid peroxidation assay.

Assay Substance	**Control 1 (C1)**	**Control 2 (C2)**	**Control 3 (C3)**	**Control 4 (C4)**	**Control 5 (C5)**	**Control 6 (C7)**
Ascorbic acid	0.1ml	-	0.1ml	-	0.1ml	0.1ml
EtOH	-	-	-	0.1ml	0.1ml	-
$FeCl_3$	0.1ml	-	0.1ml	-	0.1ml	0.1ml
Liposomes	-	-	0.2ml	0.2ml	0.2ml	0.2ml
PBS	0.7ml	0.9ml	0.5ml	0.7ml	0.5ml	0.5ml
Test substance	0.1ml	0.1ml	0.1ml*	-	-	0.1ml (PpG)**

* Added immediately before TBA test.

** PpG (10μM) added instead of test substance

Table 6.2. Purpose of controls for phospholipid peroxidation assay.

Control	**Purpose**
C1	Control for the test substance interacting/complexing with the reagents, in the absence of liposomes
C2	Control to identify the effect of the test substance on the reagents (by comparing with C1), in the absence of liposomes
C3	Control for the test substance interfering with the TBA test
C4	Control for any previous peroxidation of liposomes, in the absence of any peroxidation inducers/inhibitors
C5	Control for the presence of EtOH
C6	Positive control: PpG

After incubation, 0.1ml BHT (**148**) solution (2%) was added (to prevent the occurrence of lipid peroxidation during the TBA (**133**) test, as BHT (**148**) is a chain breaking anti-oxidant), followed by 0.5ml TBA (**133**) (1%) and 0.5ml HCl (25%).

After vortex mixing tubes were incubated at 90°C for 20min, to enable the reaction of MDA (**134**) with TBA (**133**) to form the $(TBA)_2$-MDA adduct. After cooling, the $(TBA)_2$-MDA chromogen was extracted into *n*-butanol by addition of 2.5ml *n*-butanol per tube (as the lipid suspension is turbid and unsuitable for spectroscopic analysis). After vortex mixing and centrifuging at 3500rpm for 20min at 26°C, the upper organic layer containing the chromogen was decanted into a cuvette, and the absorbance measured at 532nm using a spectrophotometer.

6.1.1.4 Data Analysis

The percentage inhibition of lipid peroxidation by the plant extracts was calculated relative to the percentage inhibition of lipid peroxidation by 10μM PpG (regarded as 100% inhibition), as follows:

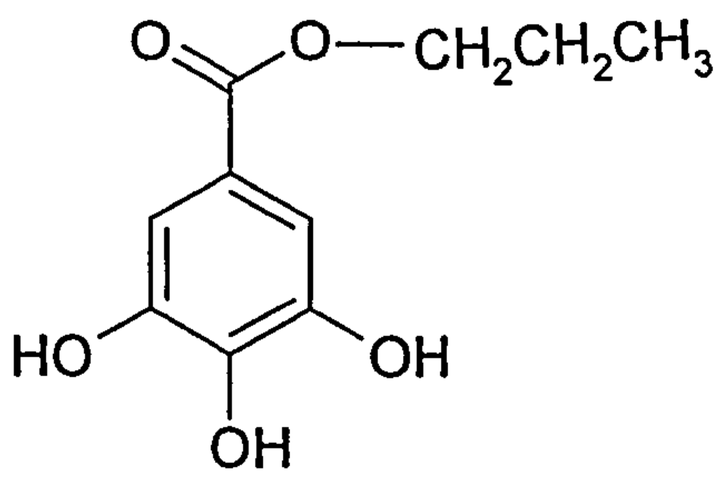
$$100 - \left[\frac{\text{extract}_{A532}}{(C5_{A532} - PpG_{A532})} \times 100 \right]$$

Data (n=3, from triplicate readings) were analysed using one-way ANOVA to determine the significance of the difference between lipid peroxidation in the absence (C5) and presence of peroxidation inducers or inhibitors (plant extracts/essential oils). Data are presented as the mean (n=3 ± SD). Significance was regarded as $p<0.01$.

6.1.2 Results and Discussion

6.1.2.1 Effect of Propyl Gallate on Inhibition of Phospholipid Peroxidation

An initial investigation was conducted to assess the effect of the antioxidant PpG (**135**) on the inhibition of lipid peroxidation. PpG (**135**) is a synthetic anti-oxidant often added to foodstuffs and is a good inhibitor of lipid peroxidation (Halliwell and Gutteridge, 1995). Concentration dependent inhibition of lipid peroxidation was observed with PpG (**135**) (Figure 6.1).



Propyl gallate (**135**)

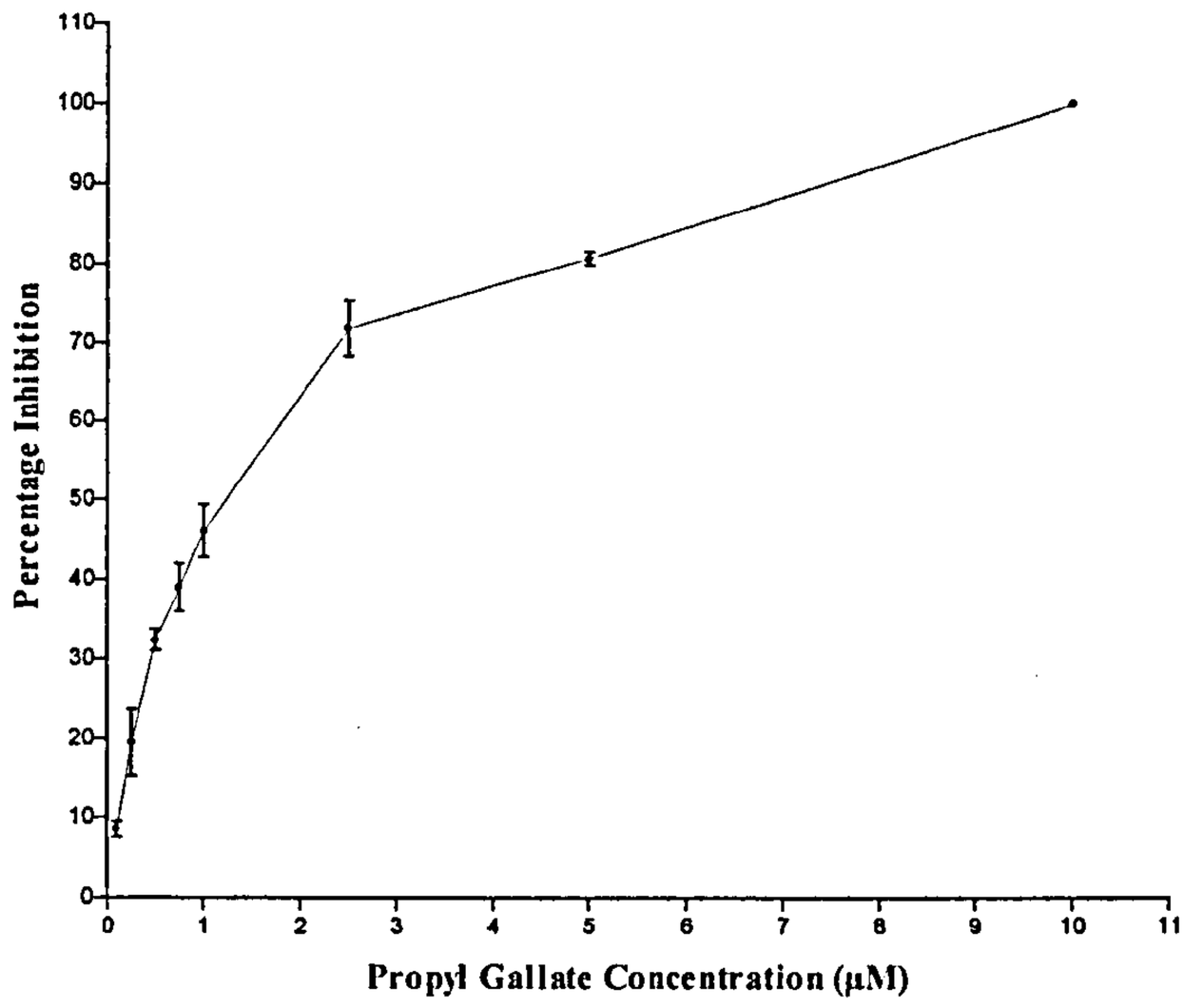


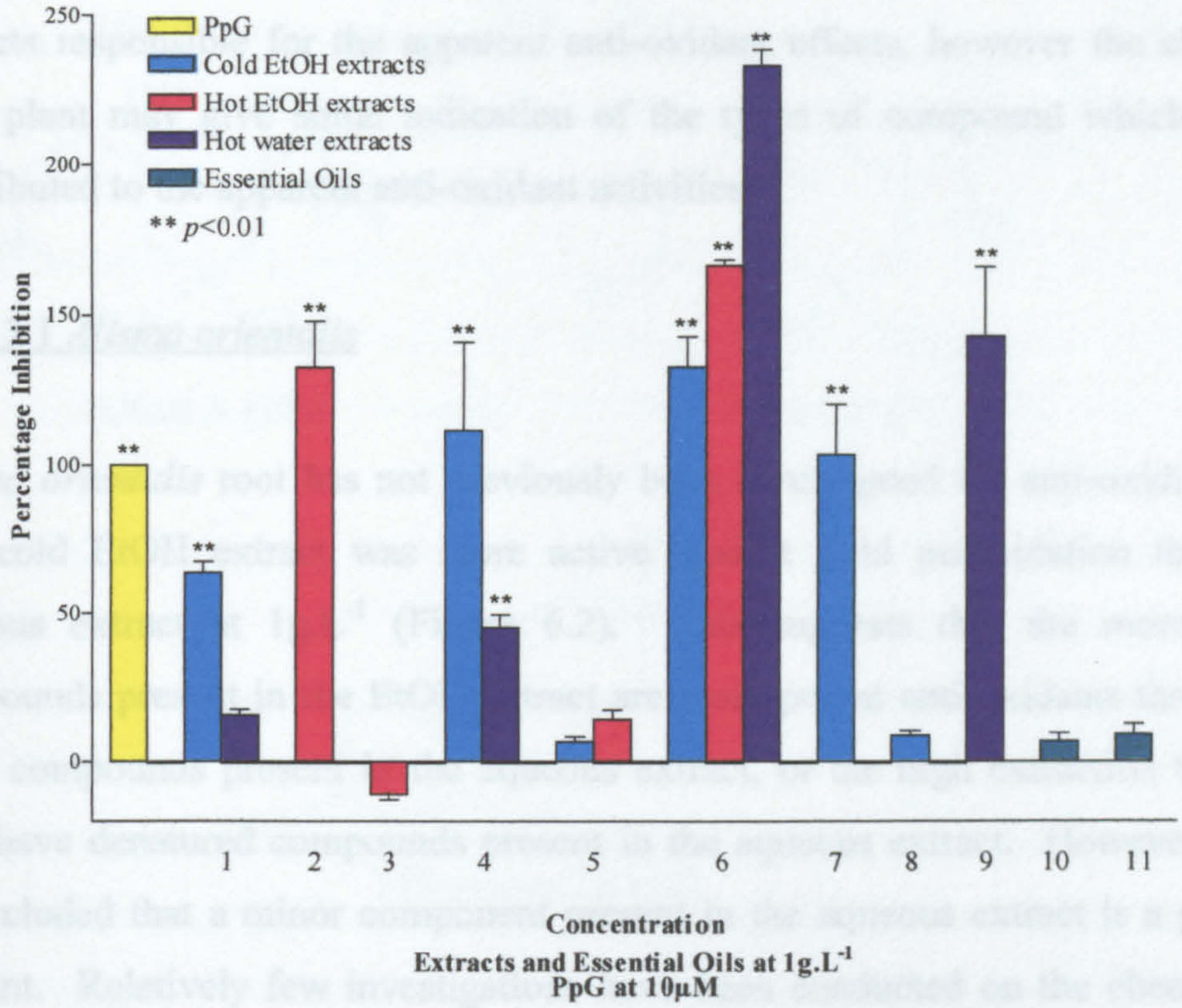
Figure 6.1. Effect of propyl gallate concentration on the percentage inhibition of lipid peroxidation of liposomes (n=3, $p<0.05$).

6.1.2.2 Effect of Plant Extracts and Essential Oils on Inhibition of Phospholipid Peroxidation

Preliminary experiments showed that some of the plant extracts inhibited lipid peroxidation *in vitro* (Figure 6.2).

The cold EtOH extracts of *Alisma orientalis*, *Polygala tenuifolia*, *Salvia miltiorrhiza* and *Withania somnifera*, the hot EtOH extracts of *Centella asiatica* and *Salvia miltiorrhiza*, and the hot aqueous extracts of *Polygala tenuifolia*, *Salvia miltiorrhiza* and *Ziziphus jujuba* (seed) significantly inhibited lipid peroxidation at $1 g.L^{-1}$ (Figure 6.2). These extracts inhibited lipid peroxidation by 63.7%, 111.3%, 132.8%, 103.5%,

132.6%, 166.4%, 45.2%, 233.2% and 143.1% respectively, compared to the control PpG (**135**) (10μM).



Key for Figure 6.2.

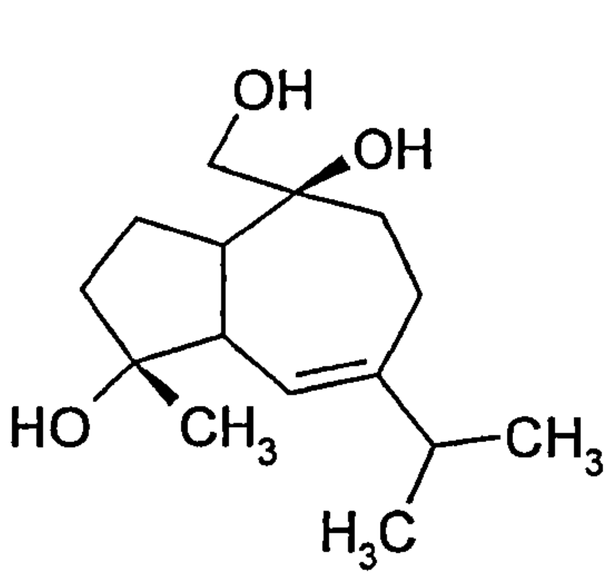
Number	Plant
1	*Alisma orientalis* root
2	*Centella asiatica* leaf
3	*Codonopsis pilulosa* root
4	*Polygala tenuifolia* root
5	Adulterated *Polygonum multiflorum* root (*Gentiana* spp.)
6	*Salvia miltiorrhiza* root
7	*Withania somnifera* root
8	*Ziziphus jujuba* fruit
9	*Ziziphus jujuba* seed
10	*Melissa officinalis* essential oil
11	*Rosmarinus officinalis* essential oil

Figure 6.2. Effect of plant extracts and essential oils on the percentage inhibition of lipid peroxidation of liposomes (triplicate readings; n=3).

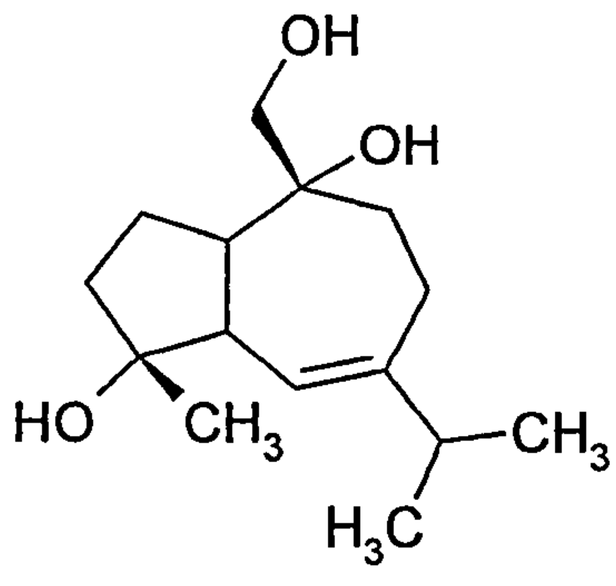
Of the extracts tested, none showed interference in the TBA (**133**) test, indicating that the effects observed were due to apparent anti-oxidant (or pro-oxidant) effects. Further investigations are required to identify the compounds in each of the active extracts responsible for the apparent anti-oxidant effects, however the chemistry of each plant may give some indication of the types of compound which may have contributed to the apparent anti-oxidant activities.

6.1.2.2.1 *Alisma orientalis*

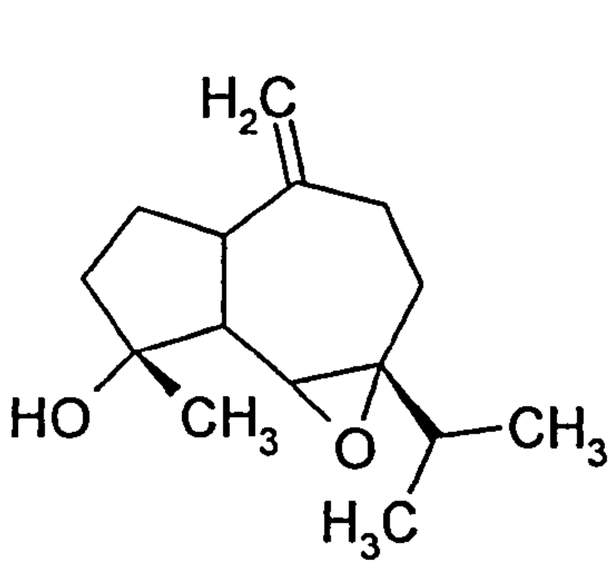
Alisma orientalis root has not previously been investigated for anti-oxidant activity. The cold EtOH extract was more active against lipid peroxidation than the hot aqueous extract at $1g.L^{-1}$ (Figure 6.2). This suggests that the more lipophilic compounds present in the EtOH extract are more potent anti-oxidants than the more polar compounds present in the aqueous extract, or the high extraction temperature may have denatured compounds present in the aqueous extract. However, it cannot be excluded that a minor component present in the aqueous extract is a potent anti-oxidant. Relatively few investigations have been conducted on the chemistry of *A. orientalis* root, and isolation of compounds that may be potential anti-oxidants is required to understand the activity of this plant.



Orientalol A (**136**)

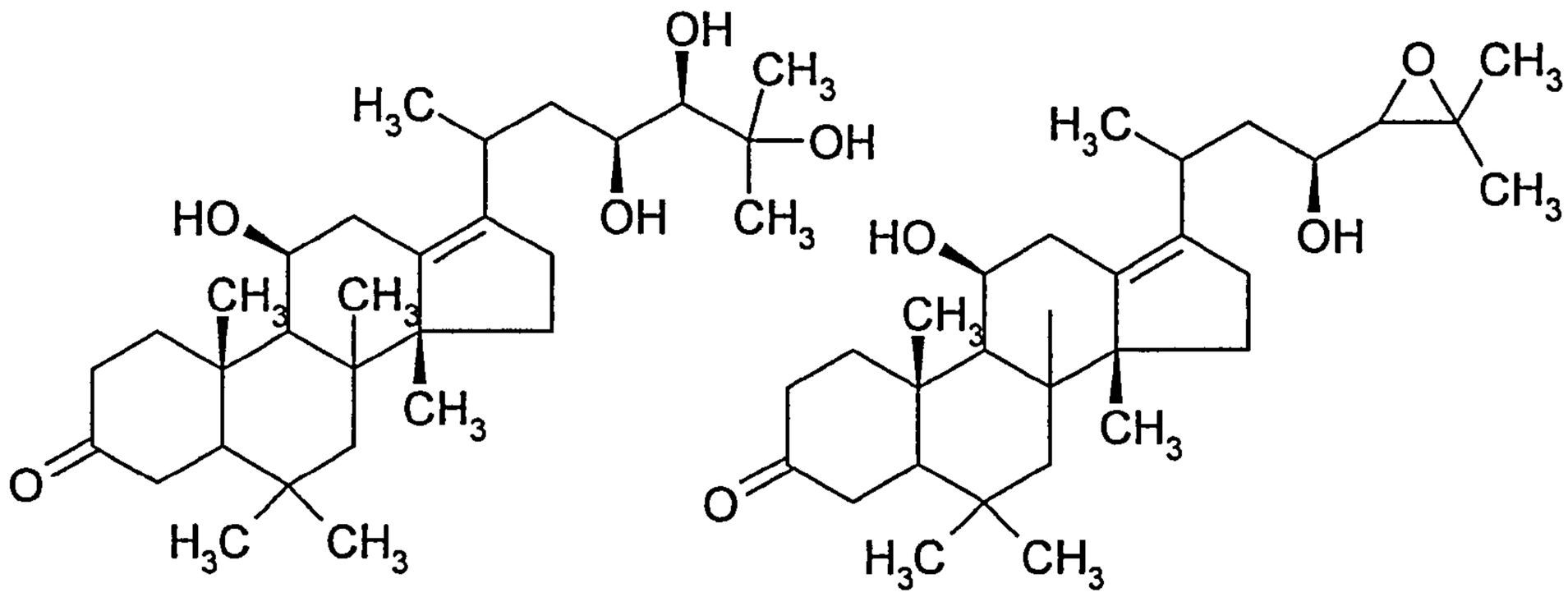


Orientalol B (**137**)



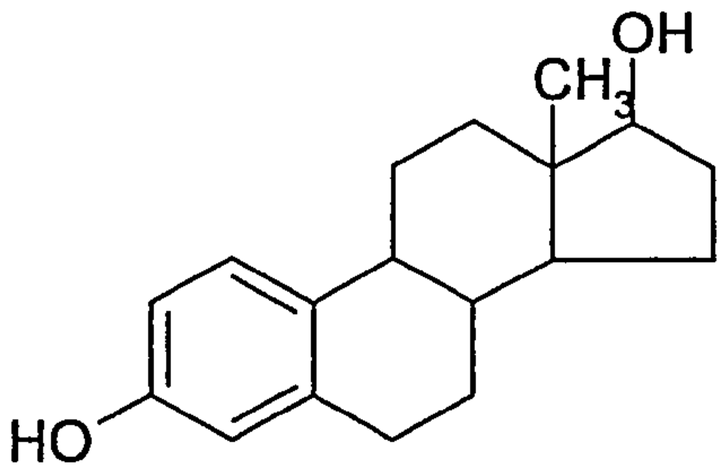
Orientalol C (**138**)

Sesquiterpenes (orientalols A (**136**), B (**137**) and C (**138**)) and triterpenes (including alisols A (**28**) and B (**30**)) have been isolated from *A. orientalis* root (Chang and But, 1987; Huang, 1993; Kamiya *et al.*, 1970; Tang and Eisenbrand, 1992; Yoshikawa *et al.*, 1992). The disesquiterpene gossypol (**59**) is a phenolic compound, which is a potent inhibitor of lipid peroxidation in rat liver microsomes (Laughton *et al.*, 1989).

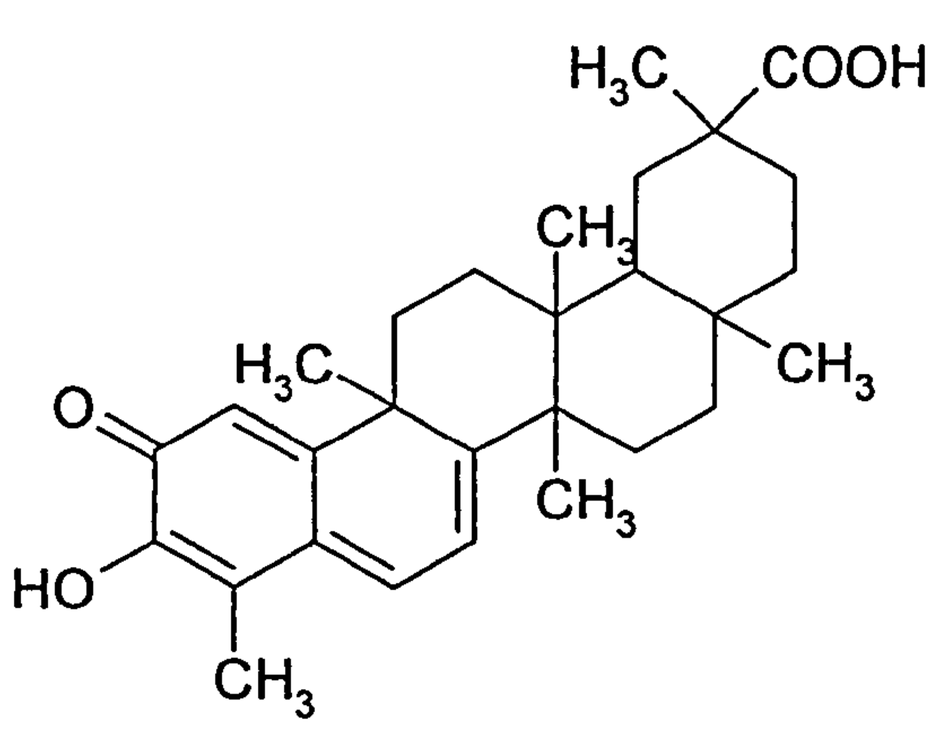


Alisol A (**28**)

Alisol B (**30**)



17β-oestradiol (**14**)



Celastrol (**139**)

The sesquiterpenes isolated to date from *A. orientalis* root are not phenolic. Some triterpenes are reported to be anti-oxidant, such as celastrol (**139**) from *Tripterygium wilfordii*, which is fifteen times more potent than α-tocopherol at inhibiting lipid peroxidation and ganoderic acids A, B, C and D from *Ganoderma lucidum* are effective inhibitors of lipid peroxidation *in vitro* (Sassa *et al.*, 1990; Zhu *et al.*, 1999). Alisols A (**28**) and B (**30**) are structurally similar to some steroidal compounds. Some steroidal compounds are reported to have anti-oxidant activity (Mooradian, 1993; Wiseman, 1994; Wiseman *et al.*, 1990). Several endogenous (e.g. 17β-oestradiol (E2)

(**14**)) and synthetic oestrogens (e.g. 17α-ethynyloestradiol) are reported to inhibit iron ion-dependent lipid peroxidation, and E2 (**14**) protects neurons against oxidative stress-induced cell death *in vitro* (Behl *et al.*, 1995; Ruiz-Larrea *et al.*, 1994; Wiseman and Halliwell, 1993). The structural similarity between anti-oxidant steroid compounds and the alisols may explain the anti-oxidant effects observed with *A. orientalis*; however, the alisols lack a phenolic component, a feature of E2 (**14**) and many plant anti-oxidants (Kimura *et al.*, 1985b; Laughton *et al.*, 1989; Okuda *et al.*, 1983). It remains to be established whether phenolic sesquiterpenes, triterpenes, or other phytochemicals are responsible for the anti-oxidant activity observed.

6.1.2.2.2 *Centella asiatica*

Centella asiatica leaf (hot EtOH extract) was significantly active against lipid peroxidation 1g.L^{-1}, and was more potent than the reference anti-oxidant, PpG (**135**) at 10μM (Figure 6.2). Tannins, flavonoids (e.g. quercetin (**104**)), sesquiterpenoid compounds, triterpenoid saponin compounds (e.g. asiaticoside (**101**)) and an essential oil, composed of sesquiterpenes and monoterpenes, are reported to occur in *C. asiatica* leaf (Asakawa *et al.*, 1982; Brinkhaus *et al.*, 2000; Günther and Wagner, 1996; Kapoor, 1990; Maquart *et al.*, 1990; Shukla *et al.*, 1999b; Sudheesh *et al.*, 1999; Vogel *et al.*, 1990).

The anti-oxidant effects of some tannins, flavonoids, saponins and essential oils is well documented, for example, the flavonoid quercetin (**104**) is reported to be a potent inhibitor of lipid peroxidation in rat liver microsomes (Dorman *et al.*, 1995; Foti *et al.*, 1996; Kelm *et al.*, 2000; Kimura *et al.*, 1985b; Laughton *et al.*, 1989; Okuda *et al.*, 1983; Torel *et al.*, 1986). The presence of these compounds may explain the anti-oxidant activity observed. Isolation and identification of flavonoids from *C. asiatica* may aid the understanding of the anti-oxidant effects of this plant; flavonoids with hydroxyl substituents on the flavonoid nucleus (e.g. quercetin (**104**)), particularly on the A and B rings, are more potent anti-oxidants than flavonoids substituted with methoxy substituents (Arora *et al.*, 1998; Cimanga *et al.*, 1999). Glycosylation of flavonoids has also been associated with a reduction of anti-oxidant activity, compared with the corresponding aglycone (Rice-Evans *et al.*, 1996).

Application of asiaticoside (**101**) from *C. asiatica* to wounds, resulted in an increase in anti-oxidant enzymes (e.g. superoxide dismutase (SOD), glutathione peroxidase)

and a decrease in lipid peroxide levels in newly formed tissue (Shukla *et al.*, 1999a). These results suggest that asiaticoside (**101**) may be anti-oxidant *in vivo*, and the observed effects on lipid peroxide levels may be due to both the increase in levels of intrinsic anti-oxidants and perhaps due to an anti-oxidant effect of asiaticoside (**101**) itself.

6.1.2.2.3 *Codonopsis pilulosa*

The hot EtOH extract of *C. pilulosa* root showed apparent pro-oxidant effects in this assay at $1g.L^{-1}$ (Figure 6.2). Some anti-oxidants, such as vitamin E, may have pro-oxidant actions under certain circumstances (Halliwell, 1990; Yamamoto and Niki, 1988) and this may occur if the substance can bind Fe (III) ions and reduce them to Fe^{2+}. Ascorbic acid may stimulate lipid peroxidation and the formation of reactive oxygen species, such as hydroxyl radicals ($^{\bullet}OH$) (Halliwell, 1990). *C. pilulosa* root may contain compounds which bind and reduce Fe (III) ions, or may contain ascorbic acid or other substances, capable of promoting formation of reactive oxygen species that increase lipid peroxidation. The potential pro-oxidant effects of this plant require further assessment in other systems to establish the mechanism of action on oxidation.

6.1.2.2.4 *Gentiana* spp. (Adulterated *Polygonum multiflorum*)

The hot and cold EtOH extracts of *Gentiana* spp. root (which was substituted for *Polygonum multiflorum* root; refer to Chapter 2, 2.2.1.1) showed only weak activity against lipid peroxidation at $1g.L^{-1}$ (Figure 6.2).

Some species of *Gentiana* have been reported to have anti-oxidant activity. *G. decumbens* showed $^{\bullet}OH$ and superoxide ($O_2^{\bullet-}$) scavenging activity *in vitro* (Myagmar and Aniya, 2000). *G. barbata* and *G. olivieri* showed hepatoprotective activity *in vivo* and prevented MDA (**134**) formation, suggesting that inhibition of lipid peroxidation occurred (Aktay *et al.*, 2000; Nikolaev, 1983). The compounds responsible for these effects have not been confirmed, but isoorientin-6'-*O*-glucoside from *G. arisanensis* showed free radical scavenging activity and inhibited lipid peroxidation *in vitro* (Ko *et al.*, 1998). Isoorientin-6'-*O*-glucoside and perhaps related compounds may have also contributed to the reported anti-oxidant effects of other species of *Gentiana*. The identification of the *Gentiana* spp. used in the present study and its chemical

constituents requires further investigation. The apparently weak anti-oxidant activity of the *Gentiana* spp. EtOH and aqueous extracts also requires confirmation in other experimental studies.

6.1.2.2.5 *Polygala tenuifolia*

The cold EtOH extract of *Polygala tenuifolia* root was more active against lipid peroxidation than the hot aqueous extract at $1g.L^{-1}$ (Figure 6.2). This suggests that the more lipophilic compounds may be more effective anti-oxidants than the more polar compounds present in the aqueous extract. It is also possible that some anti-oxidant compounds present in the aqueous extract may have been denatured by the high extraction temperature, rendering them inactive.

The traditional Chinese prescription, DX-9386, which consists of four herbs including *P. tenuifolia*, improved learning behaviour in senescence accelerated mice (SAM) and inhibited the increase in lipid peroxide in the liver and serum in SAM (Nishiyama *et al.*, 1994a). *P. tenuifolia* root alone has not previously been reported to contain potential anti-oxidants. Constituents isolated from the root of this herb include saponins (including onjisaponins A - G and tenuifolin (**67**)), phenolic glycosides (tenuifolisides A - F), fixed oil and resin (Chang and But, 1986; Huang, 1993; Ikeya *et al.*, 1991b; Sakuma and Shoji, 1981a; Sakuma and Shoji, 1981b; Tang and Eisenbrand, 1992; Trease and Evans, 1996).

Saponins are reported to reduce the hepatotoxicity of carbon tetrahydrochloride, which may be due to the deactivation of oxygen free radicals (Abe *et al.*, 1981); the saponin Rb_1, a saponin from *Panax ginseng*, is an inhibitor of lipid peroxidation (Deng *et al.*, 1990). Saponins are also structurally related to anti-oxidant steroidal compounds (e.g. E2 (**14**)), which may also explain the anti-oxidant effects observed. The phytochemicals responsible for the apparent anti-oxidant effects may be either saponins, the phenolic compounds or other unidentified compounds.

6.1.2.2.6 *Salvia miltiorrhiza*

All three extracts from *Salvia miltiorrhiza* root demonstrated significant activity against lipid peroxidation at $1g.L^{-1}$ (Figure 6.2). Of these extracts the hot aqueous extract was the most potent, which suggests that the most active compounds from the

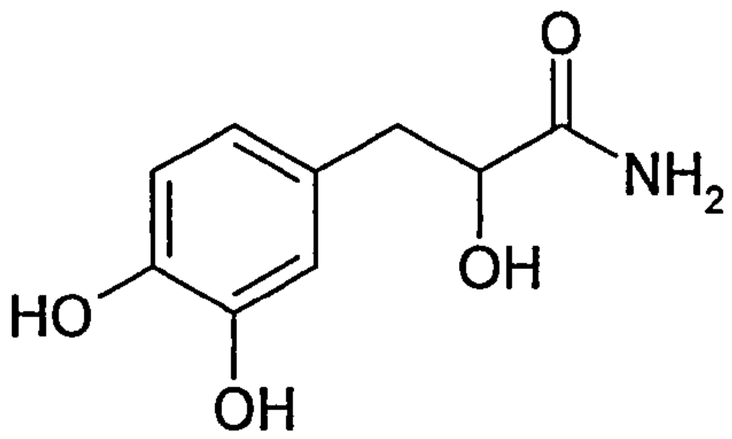
root are polar. The hot EtOH extract was more active than the cold EtOH extract; this may be due to the hot extraction process yielding more active compounds from the root. It cannot be excluded that the EtOH extracts may contain more potent anti-oxidants than the aqueous extract, but at a lower concentration. Therefore, isolation and comparison of anti-oxidant activity of compounds from both EtOH and aqueous extracts is required.

Previous reports support these findings. *S. miltiorrhiza* root protects against peroxidative damage to biomembranes (Liu *et al.*, 1992). This effect was attributed to several phenolic compounds, including the water-soluble salvianolic acids A (**123**) and B (**124**); these compounds also inhibited lipid peroxidation in rat brain, liver and kidney microsomes *in vitro* (Huang and Zhang, 1992; Liu *et al.*, 1992). Salvianolic acid A (**123**) also reduced MDA (**134**) levels in the cortex, hippocampus and corpus striatum following cerebral ischaemia-reperfusion in rats, *in vivo* (Guanhua and Juntian, 1997). The potency of these polar compounds against lipid peroxidation supports the results from these *in vitro* experiments, which assessed lipid peroxidation in bovine brain liposomes.

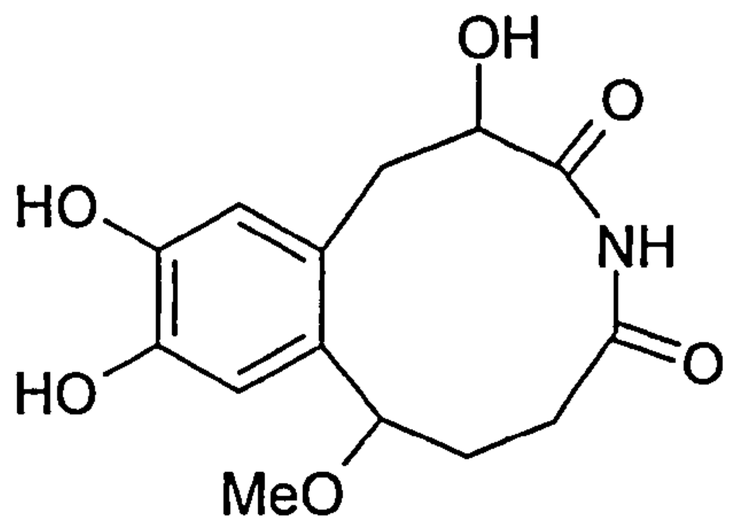
Several other investigations in various assay systems have been conducted on the anti-oxidant effects of *S. miltiorrhiza*. Dimethyl lithospermate and 3-(3, 4-dihydroxyphenyl) lactamide (**140**) (phenolic compounds) from a MeOH extract of *S. miltiorrhiza* are more potent scavengers of the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical than ascorbic acid; 2, 10, 11-trihydroxy-8-methoxy-1, 6, 7, 8-tetrahydro-2H-benzo[e]azecine-3, 5-dione (**141**) (a cyclic phenyl lactamide) from *S. miltiorrhiza* is also a scavenger of the DPPH radical; the water soluble compound danshensu (**45**) (also a phenolic compound) from *S. miltiorrhiza* is a scavenger of $O_2^{\bullet-}$ radicals, and the less polar tanshinone I (**115**) scavenges free radicals generated from lipid peroxidation of rabbit myocardial mitochondrial membranes; the root extract is active against human low density lipoprotein (LDL) oxidation; in rats with ischaemic brain injury, treatment with *S. miltiorrhiza*, increased cerebral SOD (an endogenous scavenger of $O_2^{\bullet-}$ radicals) activity and decreased cerebral MDA (**134**) levels; (Choi *et al.*, 2001; Kang *et al.*, 1997; Peigen *et al.*, 1996; Xiaoshu *et al.*, 1994; Zhao *et al.*, 1996).

Quinones, including dehydrorosmariquinone, rosmariquinone, miltirone I, tanshinone I (**115**), cryptotanshinone (**117**), dihydrotanshinone and methylenetanshinquinone, isolated from *S. miltiorrhiza* root were anti-oxidant in heated lard, but tanshinone IIa

(**116**) did not exhibit anti-oxidant activity (Weng and Gordon, 1992; Zhang *et al.*, 1990).



3-(3, 4-Dihydroxyphenyl) lactamide (**140**)

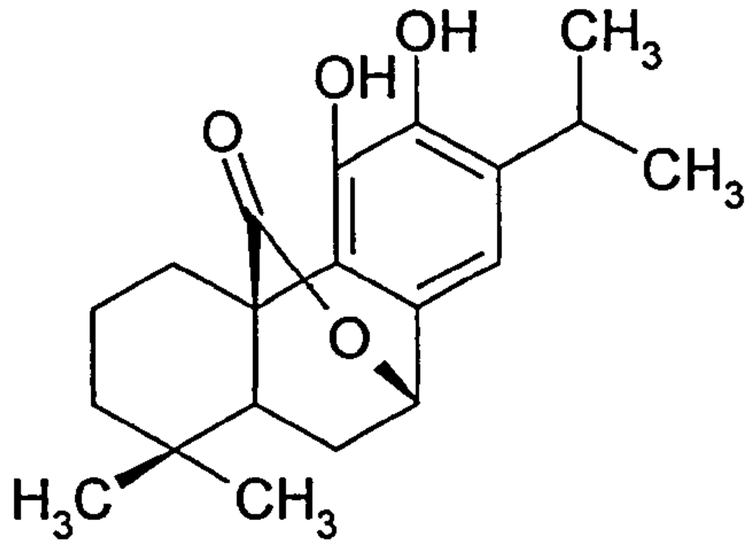


2, 10, 11-Trihydroxy-8-methoxy-1, 6, 7, 8-tetrahydro-2H-benzo[e]azecine-3, 5-dione (**141**)

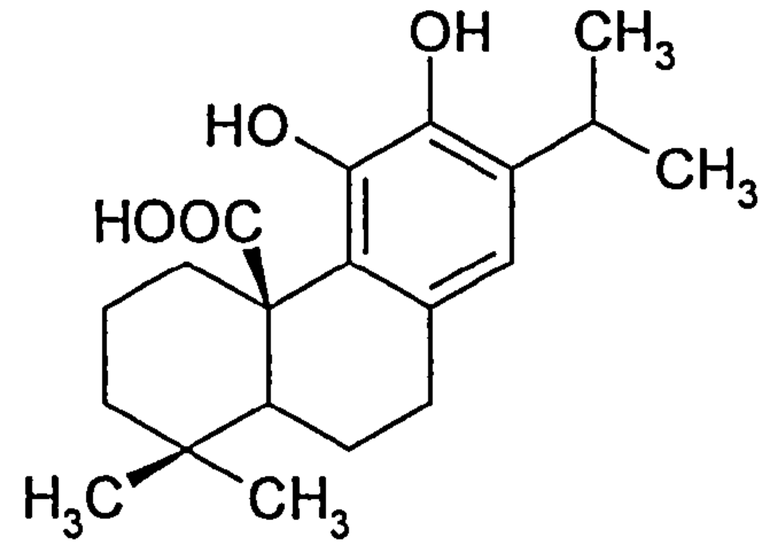
Other species of the genus *Salvia* are also reported to have anti-oxidant effects (Cuvelier *et al.*, 1996; Deans and Simpson, 2000; Hohmann *et al.*, 1999; Malenčić *et al.*, 2000; Yildirim *et al.*, 2000). Compounds from *S. officinalis* leaves which are anti-oxidant include diterpenes (e.g. carnosol (**142**), carnosic acid (**143**), methyl carnosate (**144**), rosmanol (**145**)), glycosidic compounds (e.g. 1-*O*-(2, 3, 4-trihydroxy-3-methly)butyl-6-*O*feruloyl-β-D-glucopyranoside), phenylpropanoids (e.g. rosmarinic acid (RA) (**108**)) and flavonoids (e.g. genkwanin, cirsimaritin) (Al-Sereiti *et al.*, 1999; Cuvelier *et al.*, 1994; Cuvelier *et al.*, 1996; Parnham and Kesselring, 1985; Pearson *et al.*, 1997; Schwarz and Ternes, 1992; Wang *et al.*, 2000).

The diterpenes carnosol (**142**), carnosic acid (**143**) and rosmanol (**145**), also from *Rosmarinus officinalis* leaves, are inhibitors of mitochondrial and microsomal lipid peroxidation, and inhibitors of $O_2^{\bullet-}$ radical production (Haraguchi *et al.*, 1995). Carnosic acid (**143**) and RA (**108**) have also been shown to act synergistically with lycopene against LDL oxidation (Fuhrman *et al.*, 2000).

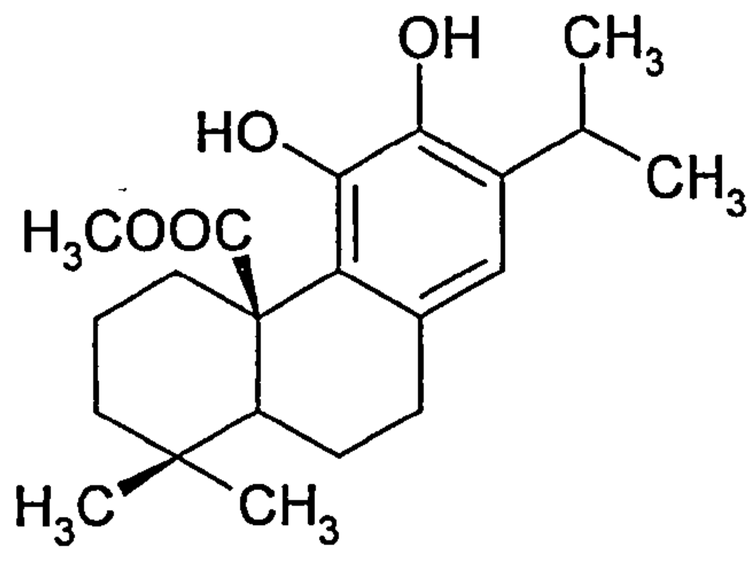
Compounds present in the *S. miltiorrhiza* extracts may also have acted synergistically against lipid peroxidation. Other compounds (e.g. flavonoid and phenolic glycosides) identified in *S. officinalis* may also have potential as anti-oxidants (Lu and Yeap Foo, 2000). *S. triloba* and *S. reflexa* extracts are also anti-oxidant (Malenčić *et al.*, 2000; Yildirim *et al.*, 2000). It is therefore apparent that the genus *Salvia* is a valuable source of various anti-oxidant compounds, which may have potential therapeutic relevance in disorders such as AD.



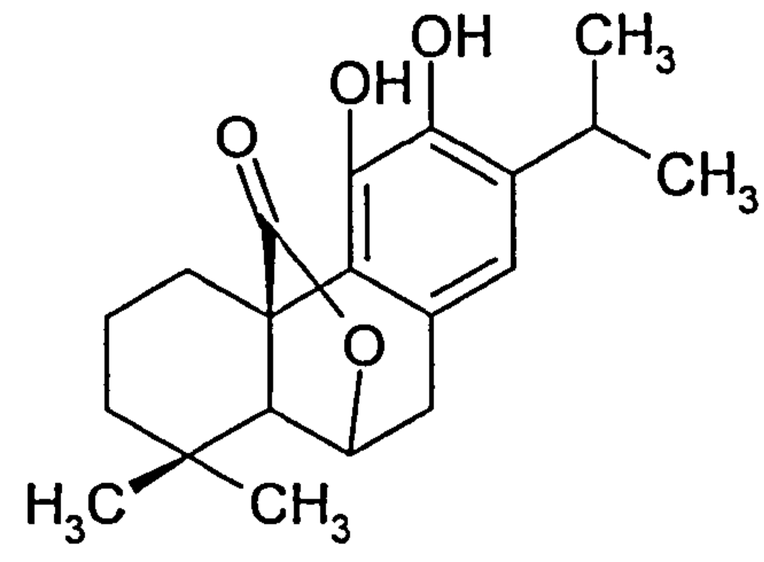
Carnosol (**142**)



Carnosic acid (**143**)



Methyl carnosate (**144**)



Rosmanol (**145**)

6.1.2.2.7 *Withania somnifera*

The cold EtOH extract of *Withania somnifera* root was also significantly active against lipid peroxidation at $1g.L^{-1}$ (Figure 6.2). The compounds responsible for these effects may be the withanolides, which are reported to have anti-oxidant activity (Bhattacharya *et al.*, 2001; Mills and Bone, 2000; Mishra *et al.*, 2000; Scartezzini and Speroni, 2000; Upton, 2000), although other unidentified compounds may also contribute to the activity observed.

The crude extract is reported to inhibit lipid peroxidation in mice liver homogenates; an aqueous extract was active against lipid peroxidation in both mice and rabbits (Dhuley *et al.*, 1998; Panda *et al.*, 1997; Panda and Kar, 1998). The glycowithanolides decreased the rise in lipid peroxidation and enhanced catalase and glutathione peroxidase activities in rat frontal cortex and striatum, induced by footshock stress (Bhattacharya *et al.*, 2001); the sitoindosides (VII - X) increased catalase, glutathione peroxidase and SOD levels in the frontal cortex and striatum of rat brain (Bhattacharya *et al.*, 1997; Bhattacharya *et al.*, 2001), indicating protective effects against free-radical damage to neurons. The root extract and the glycowithanolides (consisting of equimolar concentrations of sitoindosides VII - X

and withaferin A (**109**)) are hepatoprotective in rats and mice, an effect attributed to the anti-oxidant activity against hepatic lipid peroxidation (Bhattacharya *et al.*, 2000; Chaurasia *et al.*, 2000c).

The leaves of *W. somnifera* contain several flavonol glycosides and phenolic compounds (Kandil *et al.*, 1994); the presence of these compounds suggests the leaves from this plant may also have anti-oxidant activity, however this is yet to be investigated.

6.1.2.2.8 *Ziziphus jujuba*

The cold EtOH extract of *Ziziphus jujuba* fruit demonstrated only weak activity against lipid peroxidation at $1g.L^{-1}$, however the aqueous seed extract was significantly more active at $1g.L^{-1}$ (Figure 6.2). This herb has not previously been reported to have anti-oxidant effects. The seeds are reported to contain flavone *C*-glycosides (e.g. swertisin (**150**), spinosin (**149**)), saponins (e.g. jujubosides A and B), triterpenoids (e.g. betulin, betulic acid) and fixed oil, and the fruit is reported to contain alkaloids (e.g. zizyphusine), triterpenes (e.g. alphitolic, oleanonoic and maslinic acids), and vitamins (e.g. ascorbic acid) (Chang and But, 1987; Tang and Eisenbrand, 1992).

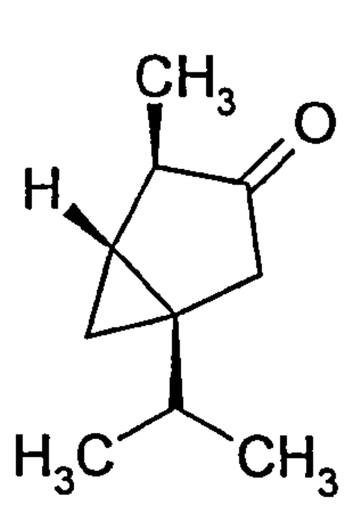
The flavonoid, saponin and triterpene content of the seeds may explain the high activity against lipid peroxidation, although these compounds and perhaps other unidentified constituents remain to be investigated for anti-oxidant activity in their isolated form. The relative inactivity of the fruit may be due to the presence of inactive or poorly active compounds, or may be explained by the presence of ascorbic acid. It is well documented that ascorbic acid may stimulate lipid peroxidation and the formation of reactive oxygen species in the presence of iron salts, and may act as a pro-oxidant (Halliwell, 1983; Halliwell, 1990). Components present may have accelerated lipid peroxidation, and perhaps counteracted any effects of anti-oxidant compounds.

6.1.2.2.9. *Melissa officinalis* and *Rosmarinus officinalis* Essential Oils

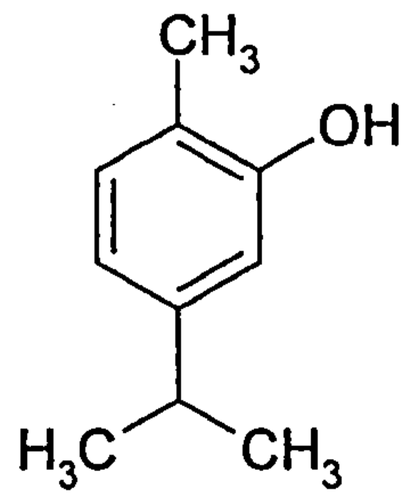
Both *Melissa officinalis* and *Rosmarinus officinalis* essential oils were weakly active against lipid peroxidation (Figure 6.2). Essential oils from various genera have been

investigated for their anti-oxidant effects; *Artemisia afra* Jacq., *Crithmum maritimum*, *Foeniculum vulgare*, *Leptospernum scoparium*, *Monarda citriodora*, *Myristica fragrans*, *Syzigium aromaticum* and *Thymus vulgaris* essential oils have shown anti-oxidant activity (Deans *et al.*, 1995; Dorman *et al.*, 1995; Graven *et al.*, 1992; Lis-Balchin *et al.*, 2000; Ruberto *et al.*, 2000). Limonene (**73**), γ-terpinene (**55**) and thymol methyl ether were the major constituents of *C. maritimum* essential oil, so may have contributed to the anti-oxidant activity (Ruberto *et al.*, 2000). γ-Terpinene (**55**) and thymol methyl ether were not detected in the *Melissa officinalis* essential oil used in the present study, and limonene (**73**) comprised <0.5% of this oil (refer to Chapter 2, 2.2.7.1).

The major components of the anti-oxidant essential oil from *A. afra* Jacq. were α- (**146**) and β-thujone, 1, 8-cineole (**56**), camphor (**57**) and α-pinene (**53**), which comprised 82% of the total oil composition (Graven *et al.*, 1992). Of these terpenes, only α-pinene (**53**) was identified in the *Melissa officinalis* essential oil used in the present study and comprised <0.5% of the total oil composition. (refer to Chapter 2, 2.2.7.1). The monoterpenes carvacrol (**147**), 6-gingerol and thymol are effective scavengers of peroxyl radicals and inhibit lipid peroxidation (Aesbach *et al.*, 1994). These compounds were also not detected in the *Melissa officinalis* essential oil used in the present study (refer to Chapter 2, 2.2.7.1), nor are they reported to occur in *R. officinalis* essential oil (Bisset, 1994; Newall *et al.*, 1996; Trease and Evans, 1996). The absence or low concentration of terpenes known to be anti-oxidant in the *Melissa officinalis* and *R. officinalis* essential oils may partly explain their apparent inactivity against lipid peroxidation.



α-Thujone (**146**)



Carvacrol (**147**)

Citrus oils and their components including geraniol (**72**) are effective scavengers of the DPPH radical (Choi *et al.*, 2000). Geraniol (**72**) comprised <1% of the *Melissa officinalis* essential oil (refer to Chapter 2, 2.2.7.1) and is not reported to be a major

constituent of *R. officinalis* essential oil, so it may be concluded that any contribution to the anti-oxidant activity of these essential oils was minimal and that geraniol (**72**) may not be a potent inhibitor of lipid peroxidation.

More than 90 essential oil constituents have been evaluated for their anti-oxidant activity by assessing their inhibitory activity against lipid peroxidation (Ruberto and Baratta, 2000). The monoterpene hydrocarbons terpinolene, α- and γ-terpinene (**55**) and phenols including thymol and carvacrol (**147**) were significantly active against lipid peroxidation, which is consistent with previous findings (Aesbach *et al.*, 1994; Ruberto and Baratta, 2000; Yanishlieva *et al.*, 1999). The alcohols including geraniol (**72**) and nerol (**75**) were also protective against lipid peroxidation, however linalool (**74**) demonstrated pro-oxidant activity (Ruberto and Baratta, 2000). Caryophyllene oxide (**78**), citral, citronellal (**68**), citronellol (**69**), α-humulene (**81**), limonene (**73**), ocimene (**76**), α-pinene (**53**) and *trans*-caryophyllene (**82**) were weakly active or inactive against lipid peroxidation (Ruberto and Baratta, 2000). These compounds comprised 47.1% of the *Melissa officinalis* essential oil, and the active compounds geraniol (**72**) and nerol (**75**) only comprised 2.1% of this essential oil (refer to Chapter 2, 2.2.7.1), which may also explain the apparent inactivity of the *Melissa officinalis* essential oil against lipid peroxidation in bovine brain liposomes. The sesquiterpenes (e.g. α-cedrene, α-humulene (**81**), *trans*-caryophyllene (**82**)) were weakly active or inactive against lipid peroxidation (Ruberto and Baratta, 2000), although further analysis of these compounds in other anti-oxidant systems would be necessary to determine any potential anti-oxidant effects.

Phenylpropanoids, also constituents of some essential oils, have been assessed for anti-oxidant activity. Eugenol (**84**) has shown good anti-oxidant activity against lipid peroxidation *in vitro* and synthetic eugenol related compounds are scavengers of $O_2^{\bullet-}$ and also inhibit lipid peroxidation (Kelm *et al.*, 2000; Lee and Shibamoto, 2000; Ogata *et al.*, 2000; Okada *et al.*, 2000; Ruberto and Baratta, 2000). The phenylpropanoid glycosides arenarioside, ballotetroside, forsythoside B and verbascoside (which do not chelate Cu^{2+}) are strong inhibitors of Cu^{2+}-induced LDL oxidation (Seidel *et al.*, 2000). Phenylpropanoids are not reported to be major constituents of *Melissa officinalis* and *R. officinalis* essential oils, which may also explain the relatively poor anti-oxidant effects observed with these oils.

The apparently weak anti-oxidant effects against lipid peroxidation may also be explained by the loss of the volatile components during the assay, such as during

incubation. This occurrence would have decreased the assay concentration. Assessment of each essential oil and the major components of each essential oil against lipid peroxidation in stoppered test tubes may minimise loss of volatile components and give a more accurate indication of the anti-oxidant activity.

It cannot be concluded that *Melissa officinalis* and *R. officinalis* essential oils are weak inhibitors of lipid peroxidation, as essential oil samples may vary considerably in composition, as highlighted by Jain *et al.* (1991), Svoboda and Deans (1992) and Vokou and Margaris (1986), due to several factors (e.g. the environmental conditions for cultivation, the age of the plant or adulteration), and samples may therefore differ in their pharmacological effects. *R. officinalis* essential oil has previously been reported to be anti-oxidant but *Melissa officinalis* essential oil was not reported to be anti-oxidant (Deans *et al.*, 1993). Further investigation in various systems for evaluating anti-oxidant activity of the isolated monoterpene, phenylpropanoid and sesquiterpene components of these essential oils to identify any potentially potent minor constituents is therefore warranted.

6.1.3 Conclusion

The CNS is rich in polyunsaturated side-chains which may be susceptible to peroxidative damage; catalase, glutathione peroxidase and SOD activity is low which suggests free-radical scavenging activity is not highly efficient; some areas of brain (e.g. substantia nigra) are rich in iron and ascorbic acid; neuronal injury may release iron, which in conjunction with ascorbic acid, promotes lipid peroxidation and $^{\bullet}OH$ radical formation (Halliwell and Gutteridge, 1995). Consequently, measurement of lipid peroxidation of bovine brain liposomes was considered the most appropriate method for the analysis of anti-oxidant activity of the plant extracts and essential oils, with regard to CNS disorders such as AD.

Assessment of inhibition of lipid peroxidation does not yield sufficient information concerning the mechanism of action of proposed anti-oxidants. In this assay, which is a metal-ion dependent system, an anti-oxidant may inhibit lipid peroxidation not only by scavenging peroxyl radicals, but may bind iron ions and stop them from accelerating peroxidation. The formation of MDA (**134**) as a result of fragmentation of cyclic- and endo-peroxides has been proposed to be inhibited by metal-ion chelators (Halliwell and Gutteridge, 1995). Either effect or both may explain the anti-

oxidant effects observed with the plant extracts. The mechanism of action may be determined by HPLC analysis, as an anti-oxidant binding to iron will not be consumed so can be detected; an anti-oxidant which scavenges peroxyl radicals will be consumed during the reaction.

Those extracts that contain flavonoids may have inhibited lipid peroxidation by metal ion chelation, as some flavonoids have demonstrated anti-oxidant activity via this mechanism (Arora *et al.*, 1998; Morel *et al.*, 1993). The presence of ascorbic acid in the assay mixture may influence the results by reducing the anti-oxidant derived radicals back to the original anti-oxidant molecule, facilitating the anti-oxidant effects. This mechanism is reported to occur if the anti-oxidant derived radicals are lipid-soluble and are accessible for reduction at the membrane surface (Halliwell, 1990). Anti-oxidant activity may therefore be greater in systems containing ascorbic acid, as has been shown with some plant phenolic compounds (Laughton, 1989).

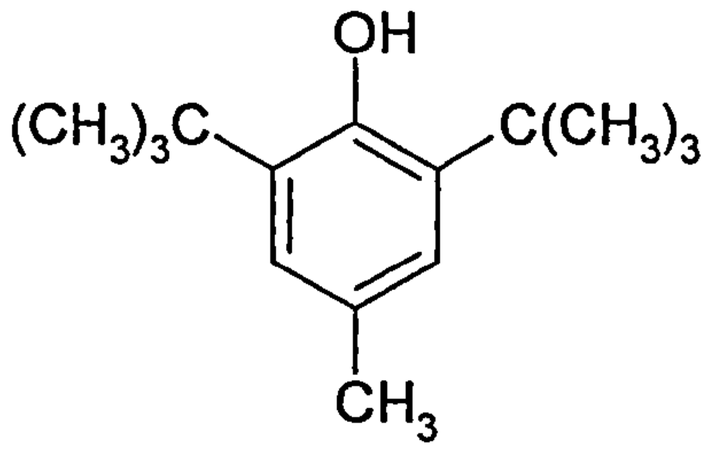
Although measurement of lipid peroxidation was considered as the most suitable assay for evaluation of anti-oxidant effects with regard to the CNS, the results cannot be regarded as conclusive. Indeed, there are limitations to the TBA (**133**) assay. Compounds other than MDA (**134**) may react in the TBA (**133**) test to form products other than the $(TBA)_2$-MDA adduct, which also absorb at 532nm, and may not reflect the inhibition of lipid peroxidation by the plant extracts. This problem may be overcome by HPLC analysis to quantify the $(TBA)_2$-MDA adduct. Only the assessment of the anti-oxidant effects of each extract in several different systems can conclusively identify potential therapeutic anti-oxidant activity. An anti-oxidant acting by inhibition of lipid peroxidation may be pro-oxidant in other systems; some plant phenolic compounds (e.g. myricetin and quercetin (**104**)) that are potent inhibitors of lipid peroxidation, may accelerate oxidative damage to DNA by reducing Fe (III) ions to Fe^{2+} or by oxidation to yield $O_2^{\bullet-}$ (Laughton *et al.*, 1989).

Activity *in vivo* is also required to determine physiologically relevant anti-oxidant effects. An anti-oxidant *in vitro* may not be anti-oxidant *in vivo* under conditions where pharmacokinetics may influence activity; similarly an inactive or pro-oxidant compound *in vitro* may be anti-oxidant *in vivo*, perhaps due to metabolic conversion to an active compound, or due to promotion of endogenous anti-oxidant substances. Other considerations with regard to AD include access across the BBB. Extracts that are more lipophilic may contain anti-oxidant constituents able to cross the BBB (e.g. the EtOH extracts of *Centella asiatica* leaf and *Salvia miltiorrhiza* root). These

extracts and their lipophilic constituents may be more active *in vivo* than the more polar aqueous extracts (e.g. the aqueous extracts of *Salvia miltiorrhiza* root and *Ziziphus jujuba* seed), as although they may contain equipotent anti-oxidants, they may not readily cross the BBB.

Potential for production of damaging anti-oxidant derived radicals, and potential toxicity of the anti-oxidant must also be assessed. Some steroidal anti-oxidants are reported to be carcinogenic (e.g. diethylstilboestrol (**15**), hexoestrol and 17α-ethynyloestradiol) (Wiseman and Halliwell, 1993). Carcinogenic potential should therefore be considered when evaluating therapeutic use of plant-derived anti-oxidants with steroidal structures. Therefore although some compounds may protect against lipid peroxidation they may be pro-oxidant *in vivo*, perhaps by causing damage to DNA. Although the isolation and identification of a pure compound may yield the development of potent anti-oxidants, this may also have disadvantages. The synergistic action of a crude plant extract may be more potent than the isolated compounds, and may provide protection against toxic effects of some constituents.

In conclusion, the apparent anti-oxidant effects of these plant extracts indicates that they may be a source of potent new anti-oxidants, and may explain their reputed effects in CNS disorders. In addition, there is an interest in the use of plant derived anti-oxidants in food and cosmetic formulations, in view of concerns raised regarding the safety of synthetic anti-oxidants such as BHT (**148**) and PpG (**135**); BHT (**148**) has been associated with hepatotoxicity and is a potential carcinogen (Ito *et al.*, 1985; Namiki, 1990; Safer and Al-Nughamish, 1999).



Butylated hydroxytoluene (**148**)

Herbal extracts of sage and rosemary have been investigated for their anti-oxidant effects in several consumables (Karpinska *et al.*, 2000; Shahidi, 2000; Wada and Fang, 1992; Wong *et al.*, 1995). Consequently the plant extracts warrant further investigation for their anti-oxidant effects, for both therapeutic and commercial purposes.

6.2 GABA Receptor Binding Assay

Radioligand receptor binding studies are a sensitive and rapid method employed to measure the interaction of ligands, including neurotransmitters and hormones, with receptors. Plant extracts, essential oils and the monoterpenes citral, geraniol (**72**) and nerol (**75**) were assessed for their effects on the inhibition of [^{3}H]-GABA binding to both $GABA_A$ and $GABA_B$ receptors *in vitro*, based on the method described by Bowery *et al.* (1983). Such investigations do have limitations, since although ligands for GABA receptors may be identified, it cannot be determined if receptor affinity is due to agonist or antagonist effects. However, receptor binding studies can be employed to aid the explanation of results from bioassays, and are useful in providing information regarding compounds which may have potential pharmacological effects *in vivo*, and which may be clinically relevant for disease management.

6.2.1 Method

6.2.1.1 Materials

Plant extracts, essential oils and monoterpenes were obtained as described previously; *Melissa officinalis* essential oil was obtained from Fragrant Earth, Taunton, England (refer to Chapter 2, 2.1.1.1).

6.2.1.2 Preparation of Membranes

The assays were conducted by Professor Norman G. Bowery, Pharmacology Department, The University of Birmingham, Edgbaston, Birmingham, as described by Bowery *et al.* (1983). In brief, the crude synaptic membranes were prepared from freshly dissected whole rat brain (from male Wistar rat) by homogenisation in 0.32M cold sucrose solution (25ml/brain), followed by successive centrifugations. The resulting crude synaptic membranes were resuspended in buffer (25ml Tris-HCl buffer (50mM) containing 2.5mM $CaCl_2$). After further centrifugation the supernatant was discarded, and the pellet reconstituted in fresh ice-cold buffer, and incubated for 15min on ice. The suspension was recentrifuged and the washing procedure was repeated twice, with 15min incubation periods between each spin. All

centrifugation was performed at 4°C. After removal of endogenous GABA (**16**) and other possible inhibitory substances by the washing process, the final pellet was resuspended in 35ml buffer prior to use in the binding assays.

6.2.1.3 Procedures for $GABA_A$ and $GABA_B$ Binding Assays

$GABA_A$ Assay

Baclofen (**20**) (62.5µl, 100µM final concentration) was added to 800µl rat synaptic membranes to block $GABA_B$ receptors. [^{3}H]-GABA (100µl, 10nM final concentration) and test substances were then added. All test substances were diluted in EtOH or DMSO prior to addition to the incubation solution and were assayed at 1µg/ml and 100µg/ml, in triplicate. Non-specific binding was determined by addition of isoguavicine hydrochloride (100µl, 100µM final concentration).

$GABA_B$ Assay

Isoguavicine hydrochloride (62.5µl, 40µM final concentration) was added to 800µl rat synaptic membranes to block $GABA_A$ receptors. $CaCl_2$ (62.5µl, 2.5mM final concentration), [^{3}H]-GABA (100µl, 10nM final concentration) and test substances were then added. All test substances were diluted in EtOH or DMSO prior to addition to the incubation solution and were assayed at 1µg/ml and 100µg/ml, in triplicate. Non-specific binding was determined by adding baclofen (**20**) (100µl, 100µM final concentration).

For both $GABA_A$ and $GABA_B$ binding assays the final mixtures were incubated for 15min at room temperature to allow binding to occur. The assay was terminated by washing with cold 50mM Tris buffer and filtered on GF/B filters; samples were then punched into vials and soaked in scintillation fluid, prior to quantitation of the radioactivity by recording CPM (10min for each measurement) using a β-scintillation counter (LS-6000 TA, Beckman Ltd.).

6.2.1.4 Data Analysis

Non-specific binding (B_{nsb}) was regarded as the binding of [^{3}H]-GABA in the presence of a receptor specific non-radioactive compound (isoguavicine hydrochloride for the $GABA_A$ assay, baclofen (**20**) for the $GABA_B$ assay). This value was subtracted from the total amount of radioactivity bound in the presence of the test substance (B_{total}) to determine the amount of test substance bound. This was expressed as a percentage of the maximum radioactivity bound in the absence of the test substance (B_{max}), and the percentage inhibition of [^{3}H]-GABA bound was calculated:

$$\text{Percentage inhibition} = 100 - \left[\frac{B_{total} - B_{nsb}}{B_{max} - B_{nsb}} \times 100 \right]$$

6.2.2 Results and Discussion

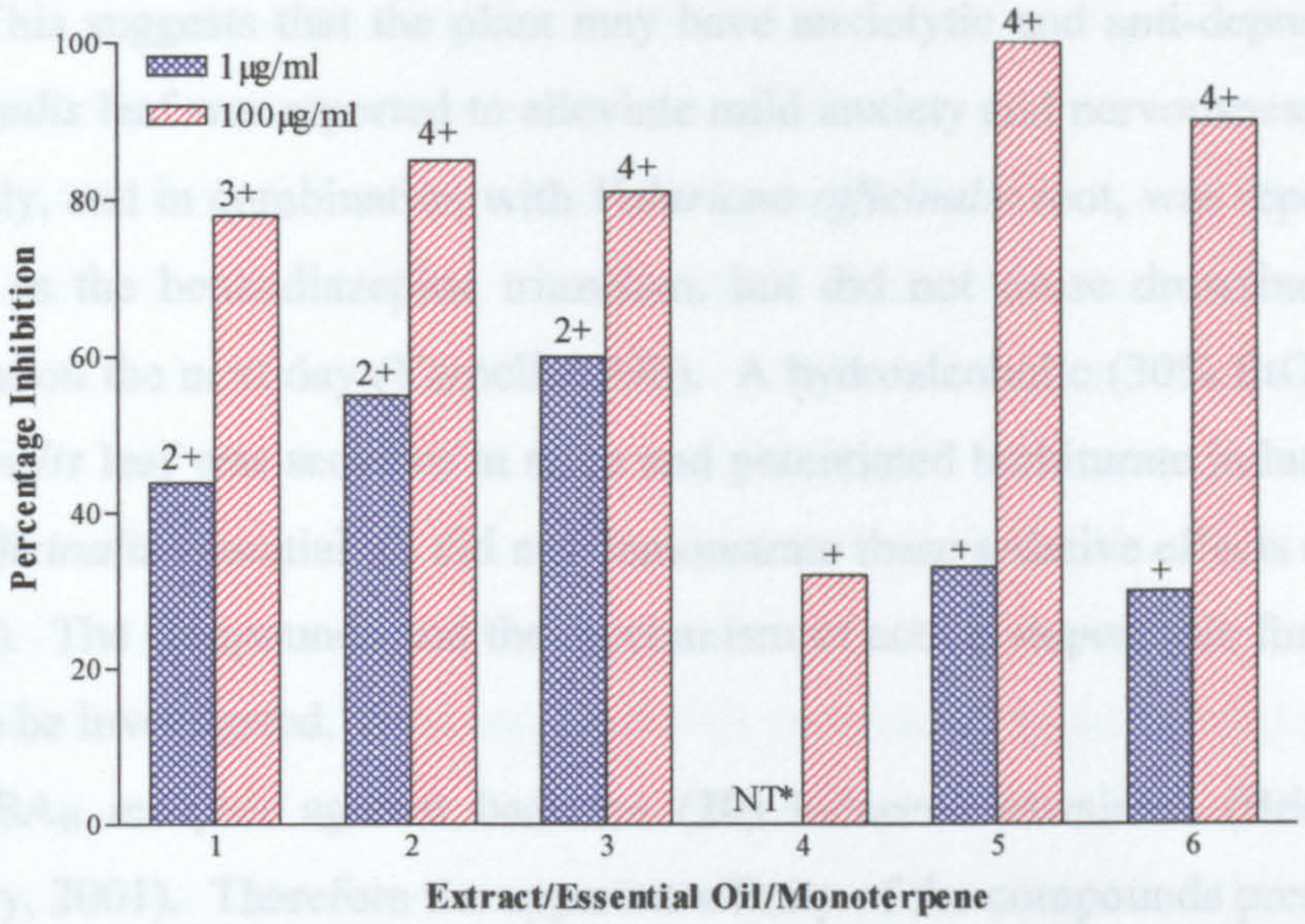
Plant extracts that tested positive for the presence of GABA (refer to Chapter 2, 2.2.6) were excluded from the receptor binding studies, as their presence in the assay systems may have produced false positive results. Therefore, those extracts tested for GABA binding effects were *M. officinalis* leaf aqueous extract, *M. officinalis* leaf EtOH extract, *Rosmarinus officinalis* dried leaf EtOH extract, *R. officinalis* fresh leaf EtOH extract, *Ziziphus jujuba* var. *spinosa* aqueous extract and *Z. jujuba* var. *spinosa* EtOH extract. *M. officinalis* and *R. officinalis* essential oils and citral, geraniol (**72**) and nerol (**75**), monoterpenes identified to be present in *M. officinalis* essential oil (refer to Chapter 2, 2.2.7.1), were also investigated.

6.2.2.1 $GABA_B$ Receptor Binding Activity of Plant Extracts, Essential Oils and Essential Oil Constituents

Test substances (at 100µg/ml) that inhibited [^{3}H]-GABA binding to $GABA_B$ receptors by ≤20% were *Melissa officinalis* leaf EtOH extract, *Rosmarinus officinalis* dried leaf EtOH extract, *Rosmarinus officinalis* fresh leaf EtOH extract, *Ziziphus jujuba* var. *spinosa* aqueous extract and *Rosmarinus officinalis* essential oil (data not shown).

6.2.2.1.1 $GABA_B$ Receptor Binding Activity of *Melissa officinalis* Leaf Extracts

The aqueous extract of *Melissa officinalis* leaf displaced [^{3}H]-GABA binding to $GABA_B$ receptors by 100% and 33% at 100µg/ml and 1µg/ml respectively (Figure 6.3).



Key for Figure 6.3

Number	Test Substance	Test Substance Type
1	Citral	Monoterpene
2	Geraniol	Monoterpene
3	Nerol	Monoterpene
4	*Melissa officinalis*	Essential oil
5	*Melissa officinalis* leaf	H_2O extract
6	*Ziziphus jujuba* var. *spinosa* seed	EtOH extract

Figure 6.3. Inhibition of [^{3}H]-GABA binding to $GABA_B$ receptors *in vitro*, by plant extracts, *Melissa officinalis* essential oil and monoterpenes. 4+, inhibition of 81% - 100%; 3+, inhibition of 61% - 80%; 2+, inhibition of 41% - 60%; +, inhibition of 21% - 40%; NT*, *Melissa officinalis* essential oil not tested at 1µg/ml.

These results suggest that polar compounds present in *M. officinalis* leaf displaced [^{3}H]-GABA binding to the $GABA_B$ receptor and that less polar compounds (present in the EtOH extract) have lower affinity for this receptor, but it cannot be excluded that a minor component of the EtOH extract has high affinity for the $GABA_B$ receptor.

In traditional medicine *M. officinalis* was used to treat neuroses and hysteria, and in Arabic medicine was used to treat depression (Bisset, 1994; Kenner and Requena, 1996). This suggests that the plant may have anxiolytic and anti-depressant effects. *M. officinalis* leaf was reported to alleviate mild anxiety and nervousness in a double-blind study, and in combination with *Valariana officinalis* root, was reported to be as effective as the benzodiazepine triazolam, but did not cause drowsiness or impair concentration the next day (Yarnell, 1998). A hydroalcoholic (30% EtOH) extract of *M. officinalis* leaf was sedative in mice and potentiated barbiturate induced sleep, but the *M. officinalis* essential oil did not demonstrate these sedative effects (Soulimani *et al.*, 1991). The compounds and the mechanism of action responsible for these effects remain to be investigated.

The $GABA_B$ receptor agonist baclofen (**20**) induces drowsiness (British National Formulary, 2001). Therefore the apparent affinity of the compounds present in the *M. officinalis* aqueous extract for the $GABA_B$ receptor may be due to agonistic effects, which may be associated with the sedative effects of this plant. If compounds were identified as $GABA_B$ agonists, this may not be appropriate for AD treatment, as such compounds are reported to impair cognitive function (Carletti *et al.*, 1993; Castellano *et al.*, 1989; McNamara and Skelton, 1996; Tang and Hasselmo, 1996). Agonistic activity may be more relevant in other disorders, such as spasticity. It cannot be excluded that $GABA_B$ antagonistic compounds also occur in the extract, so may also explain the apparent receptor binding. Their presence may be relevant in memory disorders such as AD, as $GABA_B$ antagonists are reported to enhance cognitive function (Carletti *et al.*, 1993; Froestl *et al.*, 1995; Getova and Bowery, 1998). Isolation of compounds from the aqueous extract is required to establish which compounds interact with the $GABA_B$ receptor, and their assessment *in vivo* may establish potential therapeutic effects.

6.2.2.1.2 $GABA_B$ Receptor Binding Activity of *Melissa officinalis* Essential Oil and Oil Constituents

Melissa officinalis essential oil displaced [^{3}H]-GABA binding to $GABA_B$ receptors by 32% at 100µg/ml (Figure 6.3). The monoterpenes, citral, geraniol (**72**) and nerol (**75**), present in *M. officinalis* essential oil (refer to Chapter 2, 2.2.7.1) displaced [^{3}H]-GABA binding to $GABA_B$ receptors by 78%, 85% and 81% respectively at 100µg/ml, and by 44%, 55% and 60% respectively at 1µg/ml (Figure 6.3).

It is apparent that the isomeric compounds geraniol (**72**) and nerol (**75**) demonstrated greater affinity for the $GABA_B$ receptor than citral, which may reflect the similar chemical structures of geraniol (**72**) and nerol (**75**). These results suggest that citral, geraniol (**72**) and nerol (**75**), which comprise 22.7% of the essential oil (refer to Chapter 2, 2.2.7.1), contributed to the apparent binding effects of *M. officinalis* essential oil. Other essential oil components require investigation for their GABA receptor binding effects. Citral, geraniol (**72**) and nerol (**75**) are not reported to be the major constituents of *Rosmarinus officinalis* essential oil. *R. officinalis* essential oil is reported to contain 1, 8-cineole (**56**) (15-30%), camphor (**57**) (15-25%), α-pinene (**53**) (up to 25%) and other monoterpenes (e.g. borneol (**58**), bornyl acetate (**51**)) as the major constituents (Bisset, 1994; Trease and Evans, 1996). This may explain why *R. officinalis* essential oil did not displace [^{3}H]-GABA binding as effectively as *M. officinalis* essential oil.

Several essential oils (and constituents) including lavender and neroli are reported to be sedative (Guillemain *et al.*, 1989; Jäger *et al.*, 1992), although the mode of action requires further investigation. Following inhalation, neroli (*Citrus aurantium*) essential oil and the constituents citronellal (**68**) and phenylethyl acetate were detected in mouse serum after one hour of exposure and were reported to be sedative in mice (Jäger *et al.*, 1992). It may be that essential oil constituents present in neroli essential oil bind to $GABA_B$ receptors agonistically to induce sedation, or may influence other neurotransmitter systems in the CNS. The apparent binding effects of *M. officinalis* essential oil and three monoterpenes to the $GABA_B$ receptor, indicates that essential oil constituents may induce sedation by this effect, however *in vivo* other mechanisms may also occur; oil constituents may influence benzodiazepine or barbiturate modulatory receptor sites, which may enhance the actions of GABA (**16**). Citronellal (**68**) only comprised 1.6% of the total *M. officinalis* essential oil

investigated in this study (refer to Chapter 2, 2.2.7.1). This compound requires investigation regarding its effect on GABA binding but its low concentration in *M. officinalis* essential oil may explain the lack of sedative effects observed by Soulimani *et al.* (1991).

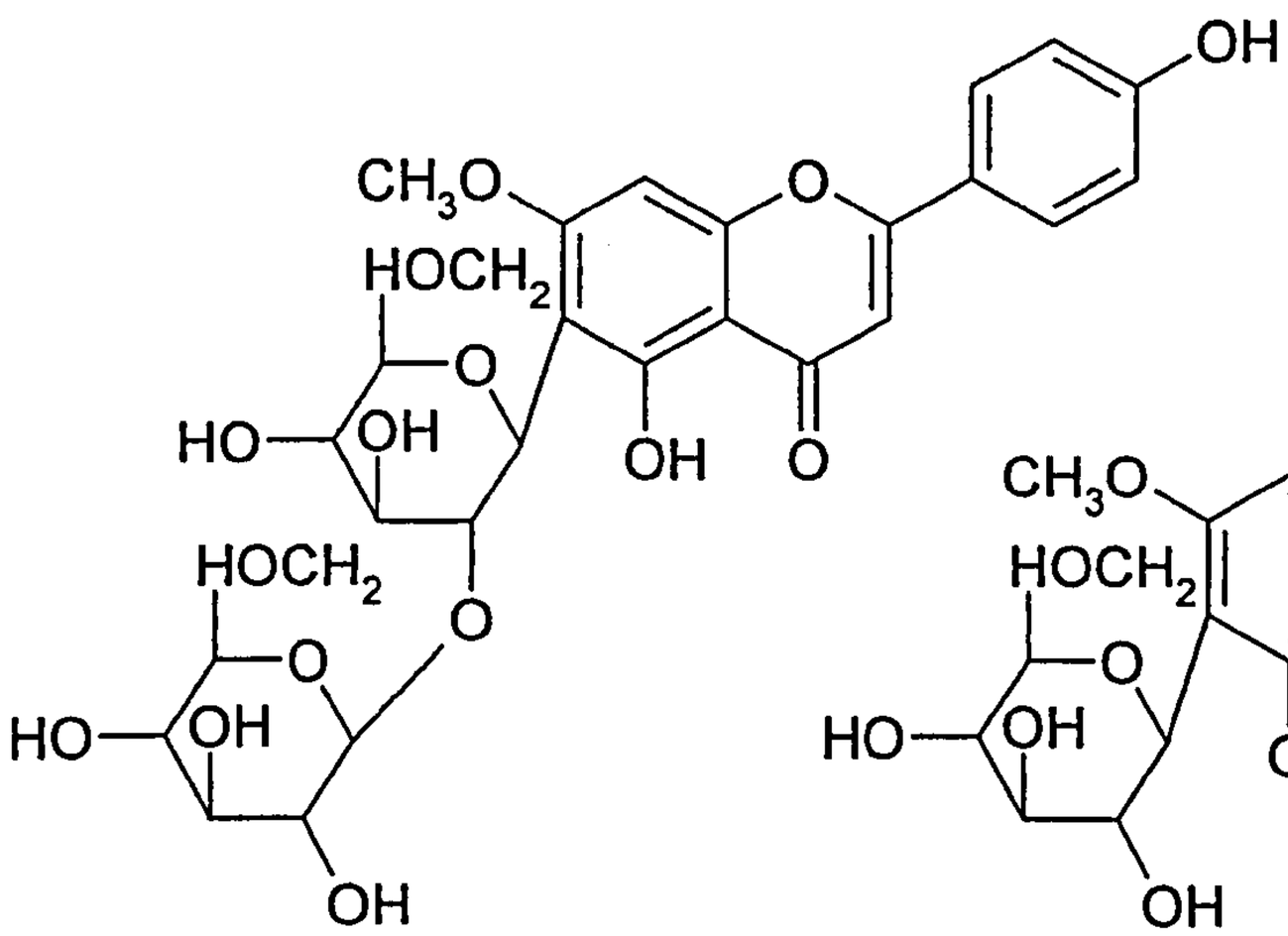
Essential oil composition may vary due to several factors (e.g. the environmental conditions for herb cultivation) so although Soulimani *et al.* (1991) found that their *M. officinalis* essential oil did not induce sedation in mice, it may still be possible that other samples of the essential oil are sedative. Indeed, the constituents caryophyllene (**82**), citral, limonene (**73**) and linalool (**74**) from a preparation of *M. officinalis* known as 'balm spirit', are reported to induce sedation in mice and the monoterpenes citronellol (**69**) and geraniol (**72**) were also weak inducers of sedation, via an unknown mode of action (Wagner and Sprinkmeyer, 1973). These compounds comprise 42.7% of the *M. officinalis* essential oil (refer to Chapter 2, 2.2.7.1) employed for assessment of GABA receptor binding. It may therefore be concluded that *M. officinalis* essential oils may vary in composition and consequently differ in their pharmacological effects, and that the sedative effects observed by Wagner and Sprinkmeyer (1973) may be due to GABA receptor modulation, as citral and geraniol (**72**) were shown to bind to GABA receptors.

M. officinalis essential oil is reported to be used in aromatherapy to alleviate depression and insomnia (McVicar, 1994). $GABA_B$ receptor activation is reported to suppress catecholamine and 5-HT release from brain slices, therefore $GABA_B$ antagonists may inhibit the suppression of NA release, induced by GABA (**16**) acting on presynaptic $GABA_B$ receptors on noradrenergic terminals (Bowery *et al.*, 1980; Pratt and Bowery, 1993). This suggests that $GABA_B$ antagonists may have potential in management of depression, and may explain the use of *M. officinalis* essential oil to alleviate depression.

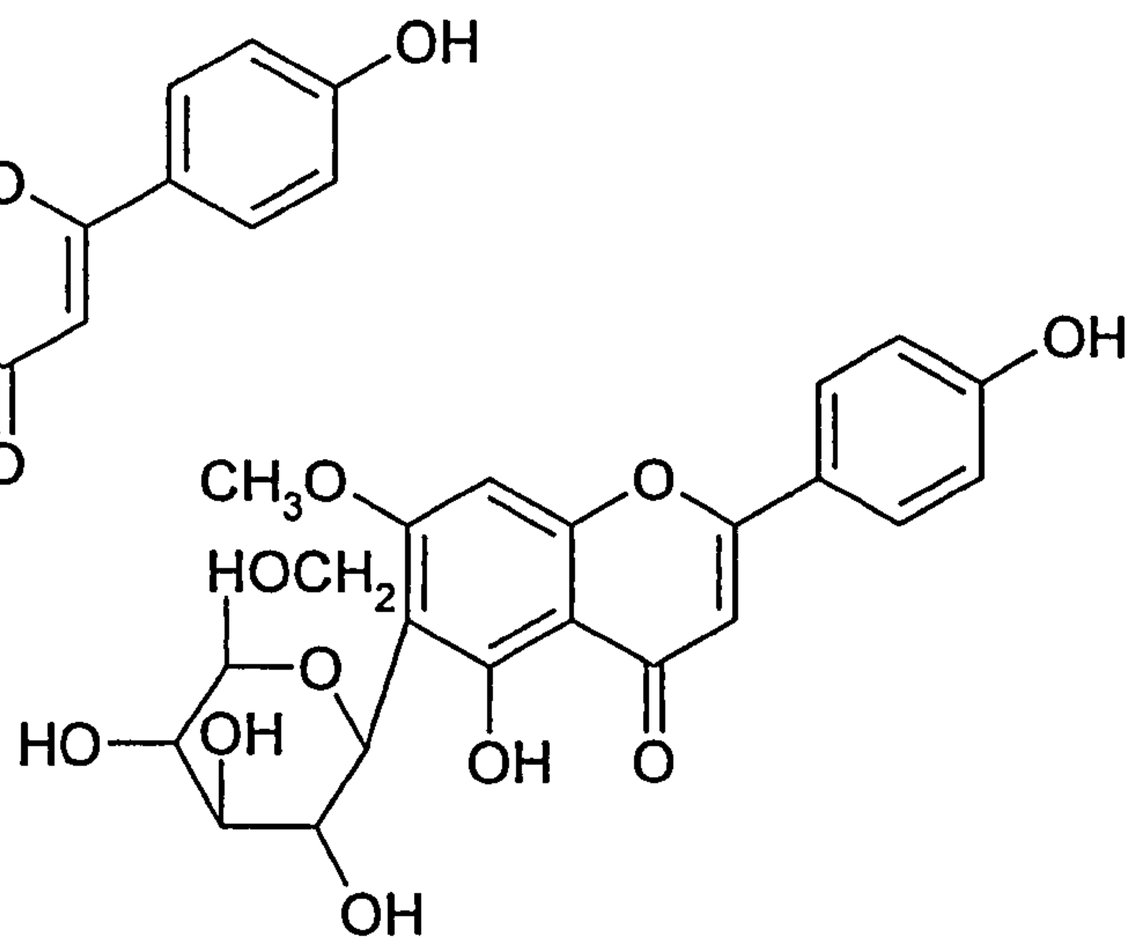
6.2.2.1.3 $GABA_B$ Receptor Binding Activity of the Ethanolic Extract of *Ziziphus jujuba* var. *spinosa* seed

The EtOH extract of *Ziziphus jujuba* var. *spinosa* seed displaced [^{3}H]-GABA binding to $GABA_B$ receptors by 90% and 30% at 100µg/ml and 1µg/ml respectively (Figure 6.3).

Z. jujuba seeds are used in traditional Chinese medicine (TCM) for anxiety and insomnia (Chang and But, 1987; Pharmacopoeia of the People's Republic of China, 1992; Tang and Eisenbrand, 1992). These effects have been substantiated experimentally; *Z. jujuba* var. *spinosa* seeds, leaves and fruit are sedative, but the pure compound jujuboside A did not influence CNS activity alone, suggesting this compound may not be responsible for sedation (Wu *et al.*, 1993). The seeds were also reported to prolong phenobarbital sodium-induced sleep in mice, and were clinically effective in treating insomnia when administered to 209 humans (Chang and But, 1987). The sedative components of *Z. jujuba* var. *spinosa* seeds have been identified as the jujubosides and flavone *C*-glycosides including spinosin (**149**) and swertisin (**150**) (Woo *et al.*, 1980; Yuan *et al.*, 1987), however these compounds may or may not be responsible for the anxiolytic effects.



Spinosin (**149**)



Swertisin (**150**)

An EtOH extract of *Z. jujuba* seeds was anxiolytic at low doses and sedative at high doses, when administered to mice (Peng *et al.*, 2000). The mechanism of action for these effects is proposed to be due to a decrease in monoaminergic activities (Hsieh *et al.*, 1986a; Hsieh *et al.*, 1986b). *Z. jujuba* seed components could also decrease monoamine levels in mouse brain (Chang and Chen, 1995). The seed extracts had no significant effects on baclofen (**20**) or muscimol (**18**) induced increase in locomotor activity in rats; it was therefore suggested that *Z. jujuba* seeds have no effect on GABAergic activity (Hsieh *et al.*, 1986b).

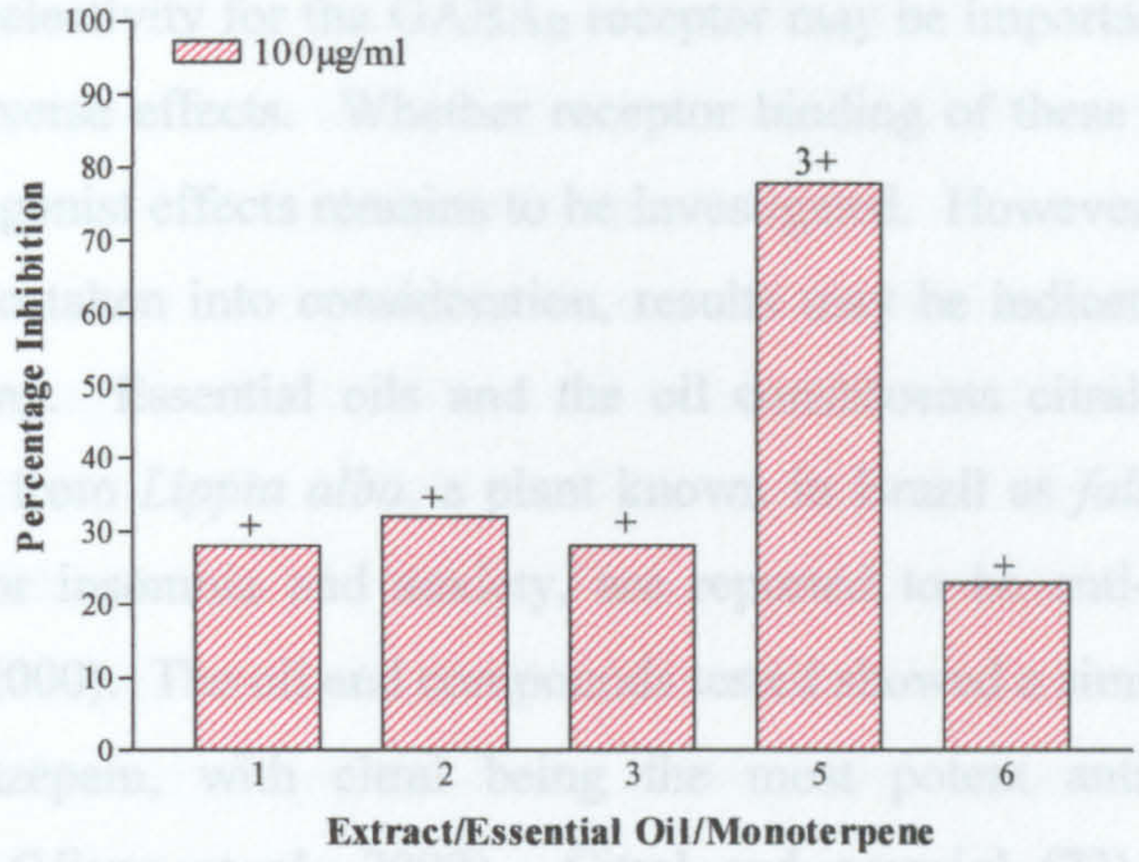
However, the results from the present investigations, showing high $GABA_B$ receptor binding, suggest that components of *Z. jujuba* seeds may promote sedation and anxiolytic effects via interaction with GABA receptors and so may have more than one mode of action. The suggestion by Hsieh *et al.* (1986b), that compounds present in *Z. jujuba* seeds do not influenece GABAergic activity, may also be explained by the compounds, which bind to GABA receptors, not reaching the CNS in sufficient concentrations, or the chemical composition of the herb used may not have contained sufficient quantities of GABA receptor binding compounds. Further studies are necessary to identify which components of the extract bind to GABA receptors and their bioavailability *in vivo*. If the sedative effects observed with this herb are associated with $GABA_B$ receptor binding, this may be agonistic.

6.2.2.2 $GABA_A$ Receptor Binding Activity of Plant Extracts, Essential Oils and Essential Oil Constituents

The affinity of some of the extracts, essential oils and oil constituents for the $GABA_A$ receptor was also investigated, to identify potential receptor selectivity. Test substances (at 100µg/ml) that displaced [^{3}H]-GABA binding to $GABA_A$ receptors by ≤20% were *Melissa officinalis* leaf EtOH extract, *Rosmarinus officinalis* dried leaf EtOH extract, *Rosmarinus officinalis* fresh leaf EtOH extract, *Ziziphus jujuba* var. *spinosa* aqueous extract and the essential oils of *Melissa officinalis* and *Rosmarinus officinalis* (data not shown).

6.2.2.2.1 $GABA_A$ Receptor Binding Activity of *Melissa officinalis* Leaf Aqueous Extract

The aqueous extract of *Melissa officinalis* leaf displaced [^{3}H]-GABA binding to $GABA_A$ receptors by 78% at 100µg/ml (Figure 6.4). Affinity of the aqueous extract for the $GABA_B$ receptor was greater than that for the $GABA_A$ receptor (Figures 6.3 and 6.4) but, as displacement of [^{3}H]-GABA binding was >70% with both receptors, the aqueous extract was not highly selective for the $GABA_B$ receptor. This may be a disadvantage in potential AD therapy, in which (antagonist) selectivity for the $GABA_B$ receptor may be a requirement.



Key for Figure 6.4

Number	Test Substance	Test Substance Type
1	Citral	Monoterpene
2	Geraniol	Monoterpene
3	Nerol	Monoterpene
5	*Melissa officinalis*	H_2O extract
6	*Ziziphus jujuba* var. *spinosa* seed	EtOH extract

Figure 6.4. Inhibition of [^{3}H]-GABA binding to $GABA_A$ receptors *in vitro*, by plant extracts and monoterpenes. 4+, inhibition of 81% - 100%; 3+, inhibition of 61% - 80%; 2+, inhibition of 41% - 60%; +, inhibition of 21% - 40%.

6.2.2.2.2 $GABA_A$ Receptor Binding Activity of *Melissa officinalis* Essential Oil and Oil Constituents

Melissa officinalis essential oil inhibited [^{3}H]-GABA binding to $GABA_A$ receptors by $\leq 20\%$, but had greater affinity for $GABA_B$ receptors (Figures 6.3 and 6.4), indicating $GABA_B$ selectivity. The essential oil constituents also showed greater affinity for the $GABA_B$ receptor (Figure 6.3), as citral, geraniol (**72**) and nerol (**75**) only displaced [^{3}H]-GABA binding to $GABA_A$ receptors by 28%, 32% and 28% respectively (Figure 6.4). *M. officinalis* essential oil and the constituents citral, geraniol (**72**) and nerol (**75**) have not previously been reported to bind to GABA receptors.

The apparent selectivity for the $GABA_B$ receptor may be important therapeutically by minimising adverse effects. Whether receptor binding of these compounds induces agonist or antagonist effects remains to be investigated. However, if pharmacological studies are also taken into consideration, results may be indicative of the nature of receptor binding. Essential oils and the oil constituents citral, myrcene (**52**) and limonene (**73**) from *Lippia alba*, a plant known in Brazil as *falsa melissa* and used traditionally for insomnia and anxiety, are reported to be anti-convulsant in mice (Viana *et al.*, 2000). The oil and compounds tested showed a similar pharmacological profile to diazepam, with citral being the most potent anti-convulsant of the monoterpenes (Viana *et al.*, 2000). Citral and geraniol (**72**) are reported to be sedative in mice (Wagner and Sprinkmeyer, 1973). Anti-convulsant and sedative effects of citral may reflect $GABA_A$ agonism, modulation of benzodiazepine receptors or other unidentified mechanisms of action. Citral binding to $GABA_A$ receptors agonistically may indicate a neuroprotective action, similar to that of the $GABA_A$ agonist muscimol (**18**) (Hollrigel *et al.*, 1996), an effect that may be exploited therapeutically. However, as citral did not show high affinity for the $GABA_A$ receptor (Figure 6.4), this theory may not be substantiated.

It is therefore possible that citral induced sedation by a benzodiazepine-like effect, or may have influenced other neurotransmitter systems. Another possibility is that sedation occurred following agonistic binding to $GABA_B$ receptors, which citral has greater selectivity for (perhaps in conjunction with weak $GABA_A$ receptor agonism). Agonistic activity at the $GABA_B$ receptor would indicate that citral and perhaps the other monoterpenes are not appropriate for AD therapy, but may provide information on the structure-activity relationships for the GABA receptors, and may act as templates for the development of new GABA agonists for management of spasticity and antagonists or partial agonists for cognitive disorders, such as AD.

6.2.2.2.3 $GABA_A$ Receptor Binding Activity of the Ethanolic Extract of *Ziziphus jujuba* var. *spinosa* seed

The EtOH extract of *Ziziphus jujuba* var. *spinosa* seed displaced [^{3}H]-GABA binding to $GABA_A$ receptors by 23% at 100µg/ml (Figure 6.4). The low affinity for this receptor suggests that compounds present in *Z. jujuba* var. *spinosa* seed may be more selective for the $GABA_B$ receptor (Figures 6.3 and 6.4). However, compounds may

also be present which have higher or equal affinity for the $GABA_A$ receptor, which warrants the isolation and identification of compounds from this herb to assess potential interactions with GABA receptors.

Z. jujuba seed extracts are reported to be anti-convulsant in animals (Chang and But, 1987), have anxiolytic effects *in vivo* and in a double blind clinical trial in humans, and were comparable to the anxiolytic effects of diazepam with fewer adverse effects (Chen *et al.*, 1986; Peng *et al.*, 2000). The mechanism for these effects has not been established. Interaction with the $GABA_A$ or $GABA_B$ receptors may explain the anti-convulsant activity and anxiolytic effects, although interaction with allosteric sites on receptors or effects on other neurotansmitter systems may explain physiological effects *in vivo*.

6.2.3 Conclusion

The results for those extracts and essential oils that exhibited binding to $GABA_A$ and $GABA_B$ receptors are consistent with the known pharmacological activities of those plants. Displacement of [^{3}H]-GABA binding to receptors may have occurred by direct interaction with the ligand binding domain (LBD) of the GABA receptor, or due to interaction with allosteric sites.

Other plants have previously been investigated for their interaction with the $GABA_A$ and $GABA_B$ receptors (Zhu *et al.*, 1996a; Zhu *et al.*, 1996b), and some plants (e.g. *Afrormosia laxiflora*, *Hoslundia opposita* Vahl. and *Salvia transsylvancia*) and compounds derived from plants (e.g. xanthone derivatives) have been identified as anti-convulsant compounds (Haruna, 2000; Maklad *et al.*, 1999; Marona, 1998; Marona *et al.*, 1998; Olajide *et al.*, 1999b), suggesting their interaction with GABA receptors. The plants investigated in this study have not previously been analysed for GABA receptor binding.

Structure-activity relationships are difficult to establish, because of the chemical diversity of compounds interacting with GABA receptors, and the complexity of physiological responses that they may initiate. The selectivity of *Melissa officinalis* essential oil and constituents for the $GABA_B$ receptor is a novel finding, as the acyclic monoterpenes citral, geraniol (**72**) and nerol (**75**) differ in their chemical structures from known ligands for the GABA receptors, such as GABA (**16**), the $GABA_A$ agonist muscimol (**18**), the $GABA_A$ antagonists bicuculline (**17**) and bis(7)-tacrine

(**19**), the cyclic $GABA_B$ receptor agonist baclofen (**20**) and the cyclic $GABA_B$ receptor antagonists phaclofen (**22**), 2-hydroxy saclofen (**23**) and the acyclic CGP 36742 (**21**). These known ligands for the GABA receptors are also all nitrogen-containing, unlike the three monoterpenes.

In conclusion, *Melissa officinalis* and its essential oil, and *Ziziphus jujuba* have potential to yield compounds, or compounds that act as templates for the development of new compounds, which may be relevant in CNS disorders involving GABAergic function such as cognitive impairment. Further investigation is required to establish the active constituents present in these herbs. Further studies would also be warranted regarding the potential agonistic and antagonistic effects of isolated compounds.

6.3 Effect of Plant Extracts on Neural Stem Cells *in vitro*

Stem cells may differentiate to give rise to a particular cell phenotype under the influence of various factors. In view of the cholinergic deficit associated with AD (Bierer *et al.*, 1995; Collerton, 1986; Giacobini, 1990; Perry *et al.*, 1978; Perry, 1986; Plotkin and Jarvik, 1986; Read, 1987), which involves the relatively selective degeneration and atrophy of cholinergic neurons (particularly in the nucleus of Meynert in the basal forebrain (Whitehouse *et al.*, 1982)), neural stem cells (NSCs) may be exploited to generate a particular neuronal phenotype for transplantation *in vivo*, as an alternative approach to the treatment of various CNS disorders.

The extracts of the plants investigated in the present study were therefore assessed for their potential effects on NSC differentiation *in vitro*, to determine if the extracts promote neuronal differentiation of a particular phenotype.

6.3.1 Method

6.3.1.1 Materials

Plant extracts were obtained as described previously (refer to Chapter 2, 2.1.1.1). Chemicals were purchased from Sigma, Fancy Road, Poole, Dorset, England, unless otherwise stated.

6.3.1.2 Isolation of Foetal Ventral Rat Forebrain Tissue and Spinal Cord Tissue, and Propagation of Neural Stem Cells *in vitro*

Isolation of foetal ventral rat forebrain tissue and spinal cord tissue, and propagation of NSCs *in vitro* was conducted by Dr Stephen Minger, King's College London, London, SE1 1UL.

Rats were housed in standard laboratory cages and had free access to food and water. Foetal ventral forebrain tissue and spinal cord tissue was isolated from pregnant Fischer 344 rats on gestational day 14 (crown-rump length: 9mm - 11mm). The rats were euthanised using a mixture of ketamine (25mg/ml), rompun (1.3mg/ml) and acepromazine (0.25mg/ml). Following removal of foetuses by laparotomy, tissue comprising the ventral forebrain with associated telencephalic ventricular zone and the spinal cord from approximately C6 - T4 was dissected.

Procedures for processing of NSCs were as described previously by Minger *et al.* (1996). Tissue from approximately 25 - 30 foetuses was collected and briefly washed in sterile Dulbecco's phosphate buffered saline (PBS; Life Technologies Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley, UK). Following a 30min incubation period at 37°C in 0.1% trypsin (in PBS), the isolated tissue was sedimented at 1000g and washed in PBS three times. Single cell suspensions were obtained by mechanical dissociation of tissue by trituration with fire-polished, narrowed Pasteur pipettes. Subsequently cell viability was evaluated using trypan blue exclusion and estimates of cell number were obtained using a haematocytometer. Single cell suspensions of isolated tissue were plated at an initial density of $2x10^4$ cells/well on $13mm^2$ glass coverslips in 24-well plates coated with 10µg/ml polyorninthine and 10µg/ml laminin (P+L) (Biogenesis Ltd., Technology Road, Poole, England). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) 1:1 F12 high glucose medium (0.6% glucose, 2mM glutamine) with N2 neuronal supplement (25µg/ml insulin, 100µg/ml transferrin, 20nM progesterone, 60µM putrescine, 30nM selenium chloride; Life Technologies Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley, UK) in humidified 95% air/5% CO_2 atmosphere. The medium was supplemented with 20ng/ml fibroblast growth factor (FGF) -2 (Chemicon International Ltd., 2 Admiral House, Cardinal Way, Harrow, England).

Once cells reached approximately 80% confluency, the medium was replaced with assay medium: DMEM/F12/N2 medium, containing 100ng/ml all-*trans* retinoic acid and plant extracts (refer to 6.3.1.3) but no serum, thus inducing cellular differentiation (Takahashi *et al.*, 1999).

6.3.1.3 Addition of Plant Extracts to Neural Stem Cells *in vitro*

Plant extracts (5mg/ml diluted in either EtOH or H_2O; refer to Chapter 2, 2.1.3 for plant extraction methods) were diluted in assay medium and added to the 24-well plates containing the NSCs, to give a final assay concentration of 25mg.L^{-1} (n=4 per plate, assay repeated >10 times). After 3 days incubation at 37°C in a humidified atmosphere (95% air/5% CO_2) the cells were processed (refer to 6.3.1.4).

6.3.1.4 Neural Stem Cell Analysis Using Immunocytochemistry

For immunocytochemical analysis, cells were washed with PBS and fixed with 4% paraformaldehyde for 30min at 4°C. Cells were washed with Tris-buffered saline (TBS; 20mM Tris, 150mM NaCl, pH 7.4); washing with TBS for 15min each time and repeated three further times. Cells were then incubated for 30min in TBS containing 0.01% Triton X-100 (TBS+). This was followed by incubation in 5% milk (in TBS+) for 30min, in order to prevent non-specific antibody binding. Cells were incubated with primary antibodies (mouse β-tubulin isotype III), diluted in 5% milk in TBS+ (1:200 dilution), at 4°C overnight.

Following overnight exposure to primary antibody, cells were washed three times in TBS+ for 15min each. Cells were then treated with horseradish peroxidase-conjugated affinipurified, goat anti-mouse IgG secondary antibody (Jackson Immunoresearch Laboratories Inc., Stratech Scientific Ltd., 61 - 63 Dudley Street, Luton, Bedfordshire), diluted in TBS+ (1:200), and incubated for 1hr at room temperature. After a further three 15min washes with TBS, cells were stained with 0.05% diaminobenzidine, 0.04% nickel chloride and 0.01% H_2O_2 (Peroxidase Substrate Kit, Vector Laboratories Inc., 30 Ingold Road, Burlingame, USA) for 8min - 10min. Cells were washed in TBS prior to being briefly dehydrated through a series of graded alcohols (70%, 95% and 100% sequentially). After passage in xylene, coverslips were mounted on glass microscope slides using DPX. NSCs were

examined using a Nikon E800 microscope equipped with the image analysis software Lucia.

6.3.2 Results and Discussion

The EtOH extracts of *Alisma orientalis* root, *Apocynum lancifolium* leaf, *Centella asiatica* leaf, *Codonopsis pilulosa* root, *Convallaria majalis* leaf, *Gentiana* spp. root (adulterated *Polygonum multiflorum* root), *Melissa officinalis* leaf, *Polygala tenuifolia* root, *Rosmarinus officinalis* leaf (fresh and dried leaf), *Salvia miltiorrhiza* root, *Withania somnifera* root, *Ziziphus jujuba* seed, and *Ziziphus jujuba* var. *spinosa* seed were assessed for their potential effects on NSC (from foetal rat forebrain) differentiation *in vitro*, at a concentration range of 12.5mg.L^{-1} - 200mg.L^{-1}.

All plant extracts were cytotoxic at concentrations 50mg.L^{-1} - 200mg.L^{-1}, except *Z. jujuba* and *Z. jujuba* var. *spinosa* seed extracts, which were only cytotoxic at concentrations ≥100mg.L^{-1}, and *Codonopsis pilulosa* and *Gentiana* spp. (adulterated *Polygonum multiflorum*) root extracts, which were not cytotoxic (data not shown). All extracts, except for *Apocynum lancifolium* leaf, *Codonopsis pilulosa* root, *Gentiana* spp. root (adulterated *Polygonum multiflorum* root), *Z. jujuba* seed, and *Z. jujuba* var. *spinosa* seed extracts, demonstrated notable cytotoxicity within the concentration range of 12.5mg.L^{-1} - 25mg.L^{-1} (data not shown). As the EtOH extracts of *Apocynum lancifolium* leaf and *Z. jujuba* var. *spinosa* seed consistently affected NSC differentiation *in vitro*, they were selected for further investigation and aqueous extracts were also assessed for their effects on NSC (from foetal rat forebrain) differentiation. Further assessment of the aqueous extracts of *Apocynum lancifolium* leaf and *Z. jujuba* var. *spinosa* seed (which were less cytotoxic than the EtOH extracts) on NSCs from foetal rat spinal cord was also conducted.

The preliminary results show that both the aqueous and EtOH extracts of *Apocynum lancifolium* leaf and *Z. jujuba* var. *spinosa* seed influence NSC (from foetal rat brain and spinal cord) differentiation at a concentration of 25mg.L^{-1} in the presence of retinoic acid (Figures 6.6 - 6.9 and 6.11 - 6.12), compared to NSC differentiation in the presence of retinoic acid alone (Figures 6.5 and 6.10).

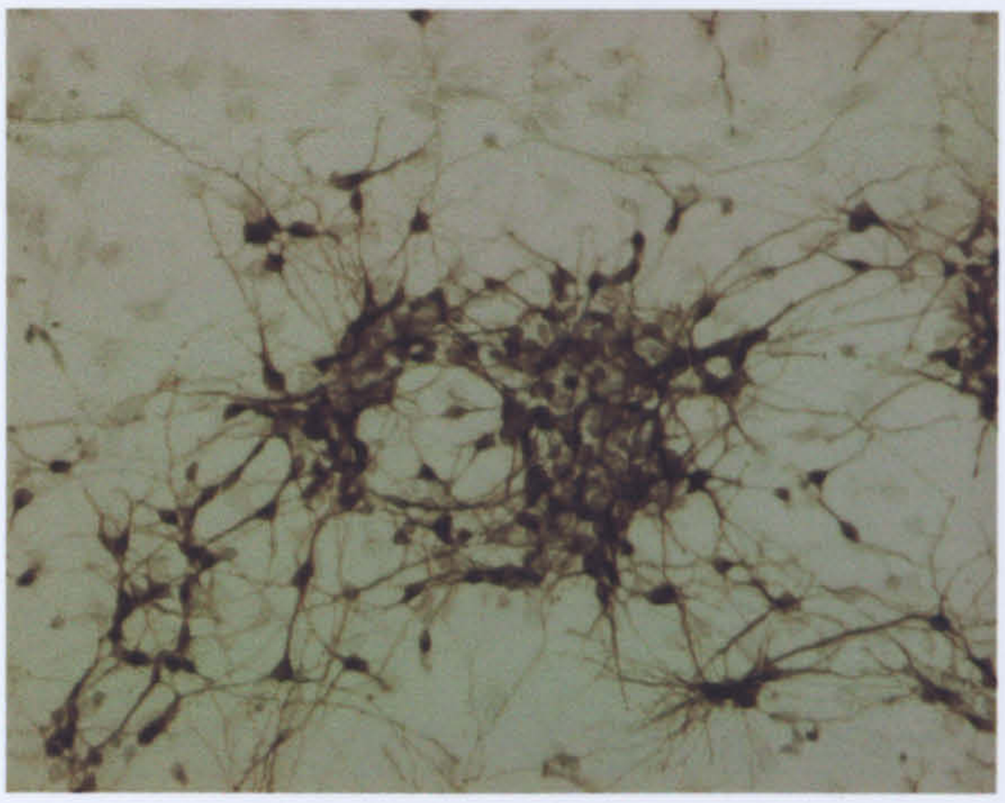


Figure 6.5. Neural stem cells from foetal rat forebrain treated with retinoic acid and stained for β-tubulin (x200).

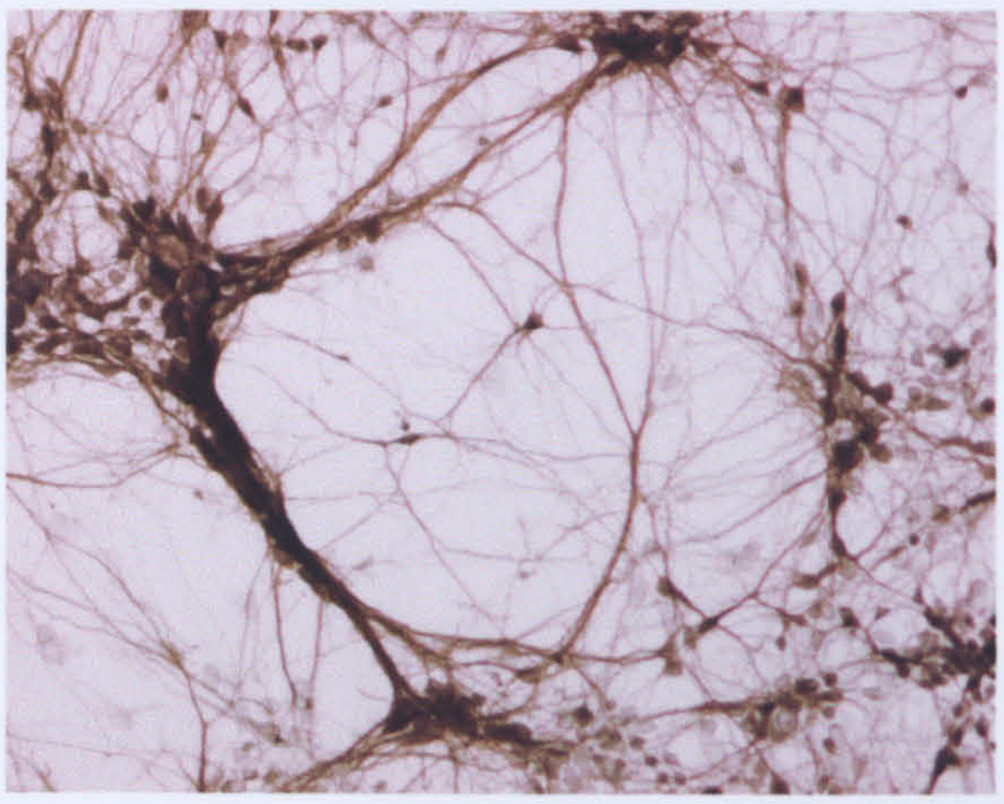


Figure 6.6. Neural stem cells from foetal rat forebrain treated with retinoic acid and *Apocynum lancifolium* leaf aqueous extract and stained for β-tubulin (x200).

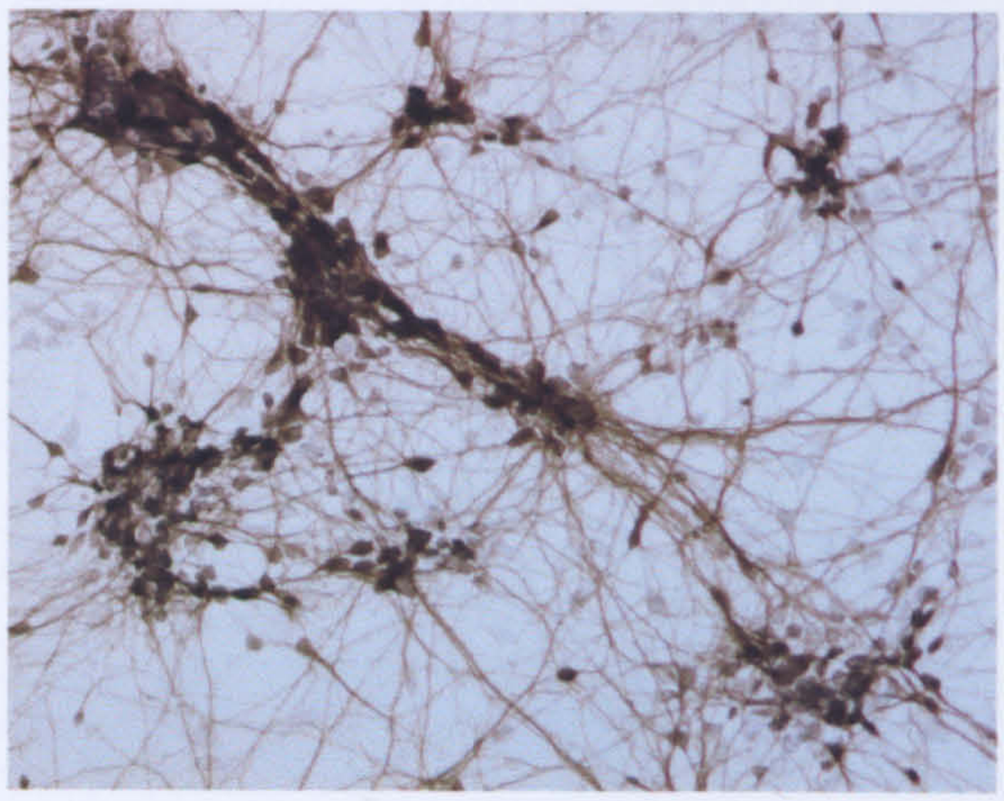


Figure 6.7. Neural stem cells from foetal rat forebrain treated with retinoic acid and *Apocynum lancifolium* leaf ethanolic extract and stained for β-tubulin (x200).

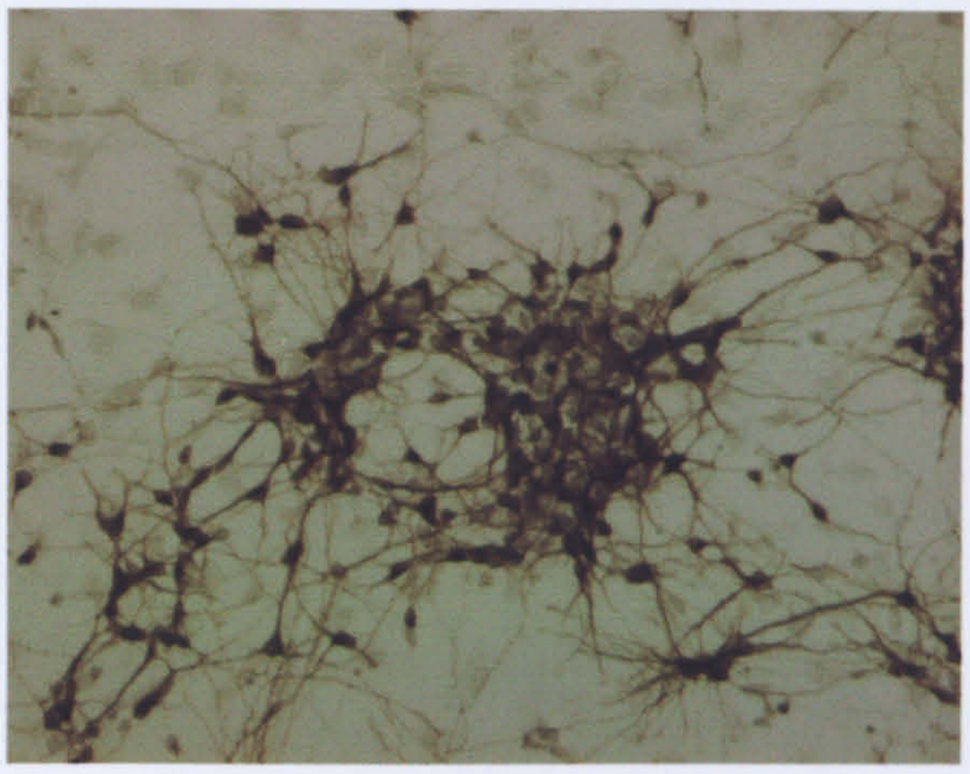


Figure 6.5. Neural stem cells from foetal rat forebrain treated with retinoic acid and stained for β-tubulin (x200).

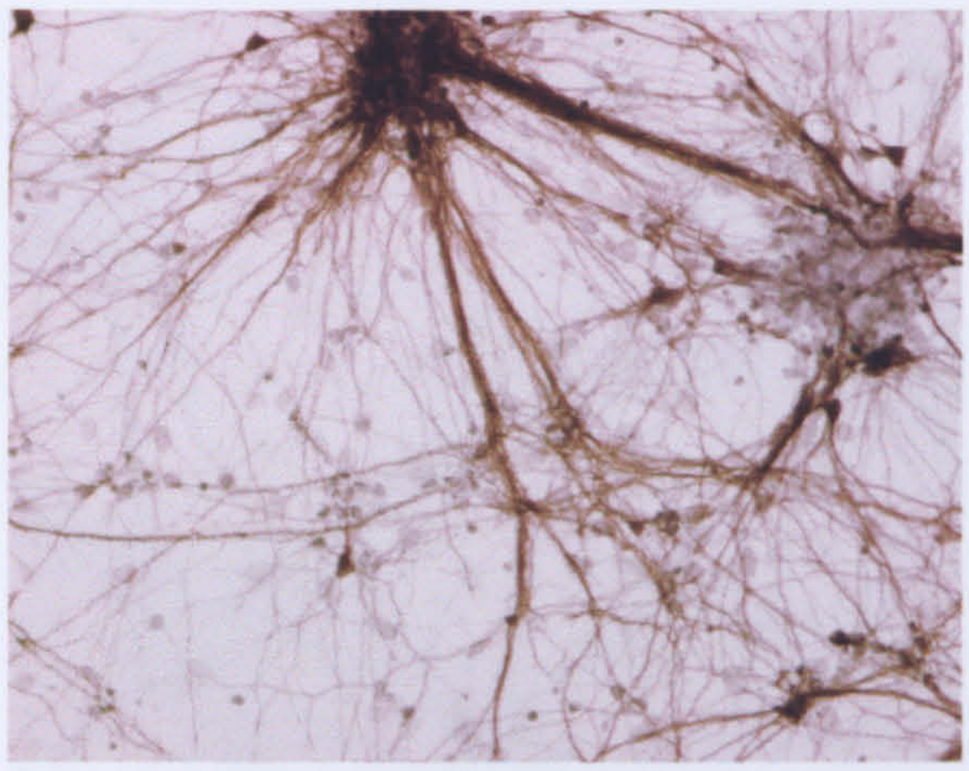


Figure 6.8. Neural stem cells from foetal rat forebrain treated with retinoic acid and *Ziziphus jujuba* var. *spinosa* seed aqueous extract and stained for β-tubulin (x200).

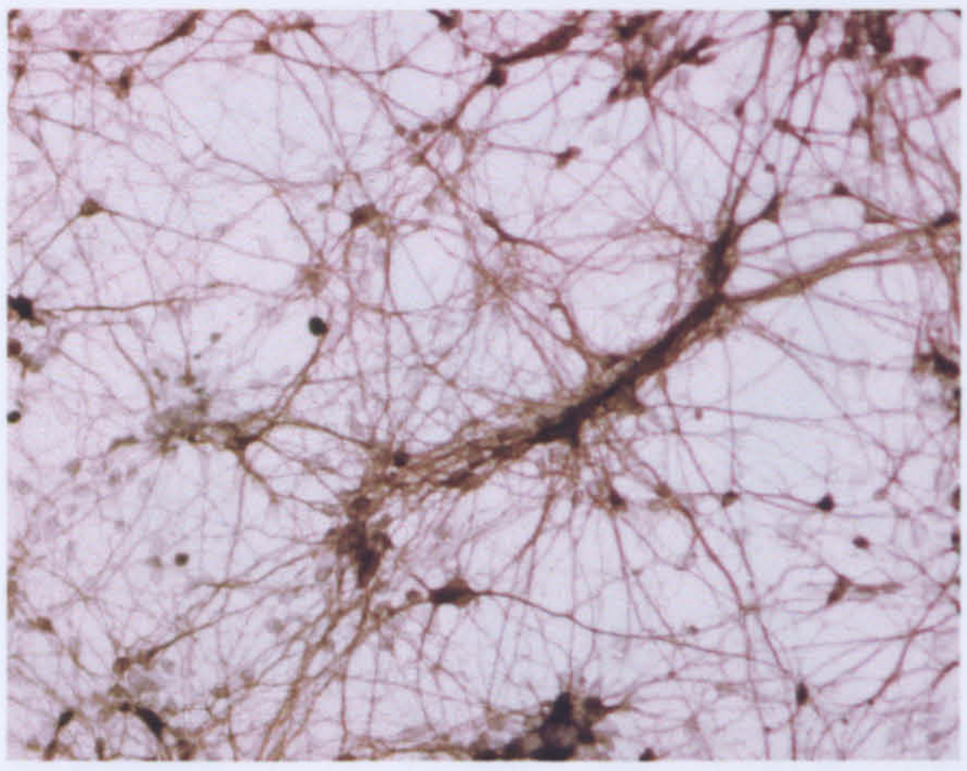


Figure 6.9. Neural stem cells from foetal rat forebrain treated with retinoic acid and *Ziziphus jujuba* var. *spinosa* seed ethanolic extract and stained for β-tubulin (x200).

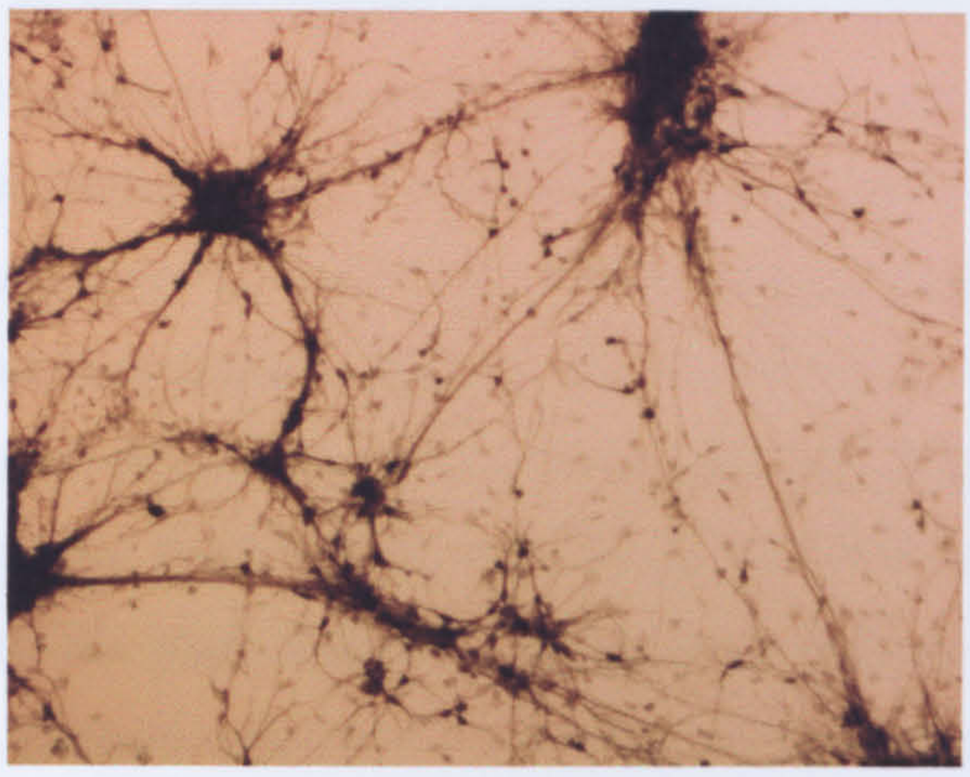


Figure 6.10. Neural stem cells from foetal rat spinal cord treated with retinoic acid and stained for β-tubulin (x200).

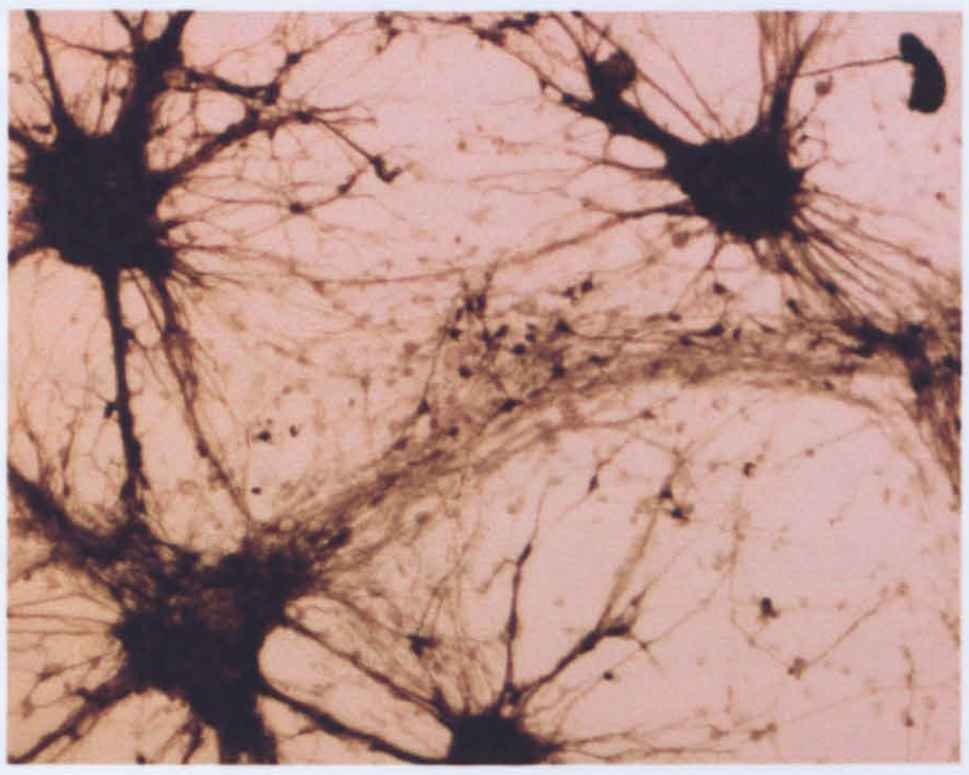


Figure 6.11. Neural stem cells from foetal rat spinal cord treated with retinoic acid and *Apocynum lancifolium* leaf aqueous extract and stained for β-tubulin (x200).

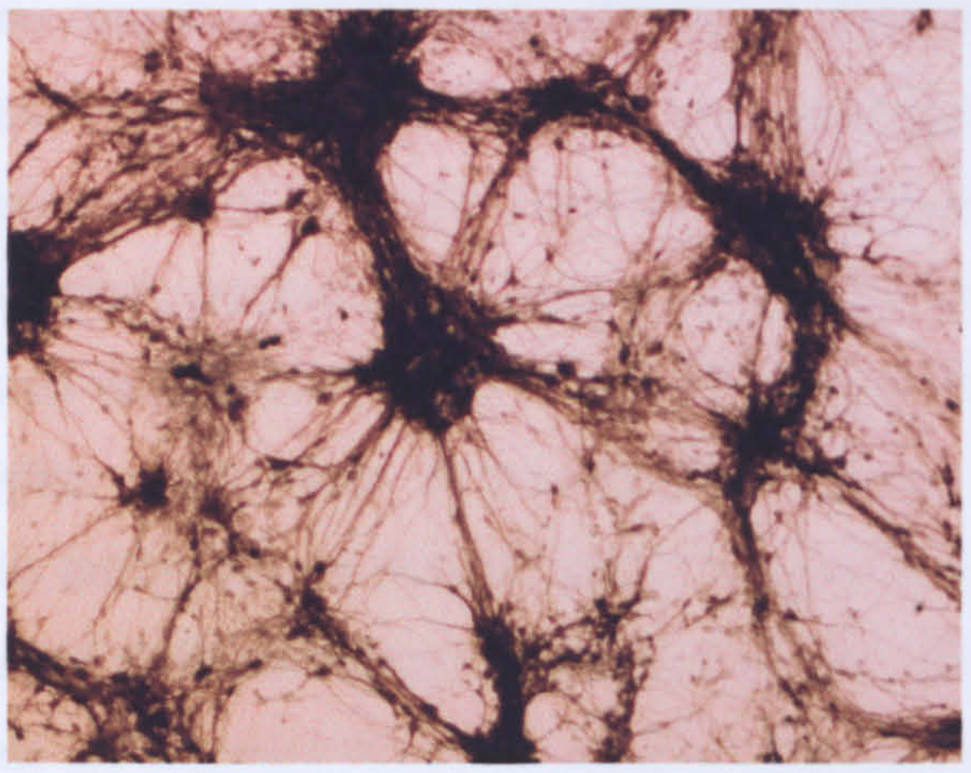


Figure 6.12. Neural stem cells from foetal rat spinal cord treated with retinoic acid and *Ziziphus jujuba* var. *spinosa* seed aqueous extract and stained for β-tubulin (x200).

This is apparent as the cells treated with the plant extracts stained positive for β-tubulin, which is regarded as a neuron-specific marker, and showed more extensive neurite elongation (Figures 6.6 - 6.9 and 6.11 - 6.12) than those cells treated with retinoic acid alone (Figure 6.5 and 6.10). Neurites also appeared to form in bundles in the presence of the plant extracts (Figures 6.6 - 6.9 and 6.11 - 6.12), as opposed to the predominantly single neurites observed in the control cells (Figure 6.5 and 6.10).

Extensive staining (for β-tubulin) of both axons and dendrites was observed in the NSCs treated with the plant extracts (Figures 6.6 - 6.9 and 6.11 - 6.12), but it is unknown if the extracts selectively promoted either axonal or dendritic neurite outgrowth; further analysis using the dendritic marker microtubule-associated protein-2 (MAP-2) and the axonal marker tau 1 would therefore be necessary.

Further experiments are also required to determine if the plant extracts promote neuronal differentiation of a specific phenotype, by conducting immunocytochemical analysis to identify any specific neuronal markers. For example, other primary antibodies may be employed including those against choline acetyltransferase (ChAT), which is specific for cholinergic neurons. Preliminary experiments were conducted using ChAT, but assay results were unsuccessful and further assay refinement will be required to establish if plant extract treated cells stain positive for cholinergic neurons. Morphological analysis of the cells treated with plant extracts shows that some cells are bipolar with triangular cell bodies; this may be indicative of cholinergic neurons, but confirmation of this is required.

The mechanism of action to explain neuronal differentiation in the presence of the plant extracts also requires further investigation. Retinoic acid is reported to potentiate cell responsiveness to neurotrophins (e.g. nerve growth factor (NGF)), and promote the development of neuronal phenotypes (Takahashi *et al.*, 1999). It is possible that compounds present in the plant extracts may have neurotrophic effects, but those compounds responsible require isolation and identification.

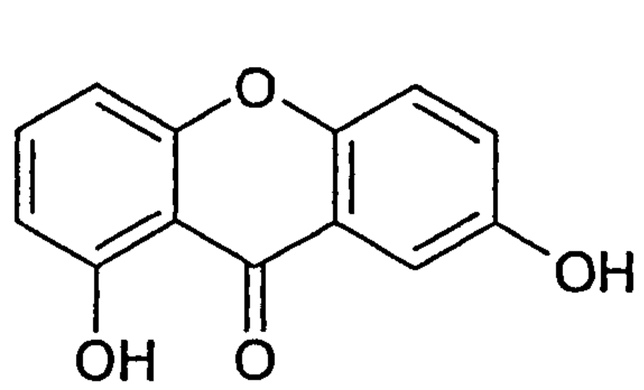
The steroid receptor superfamily of transcription factors includes receptors for oestrogens, thyroid hormone and retinoic acid. Thyroid hormone and retinoids are reported to influence CNS development (Barres *et al.*, 1994) and basal forebrain cholinergic neurons are reported to have oestrogen (estrogen) receptors (ERs) that are co-localised with NGF receptors (Sohrabji *et al.*, 1994; Toran-Allerand *et al.*, 1992). The active plant extracts may influence neuronal differentiation by modulating the function of these receptors. For example, *Polygala tenuifolia* was previously reported

to increase NGF secretion *in vitro* (Yabe *et al.*, 1997); this effect may be associated with its oestrogenic activity (observed in yeast and mammalian cells; refer to Chapter 4, 4.5). The possibility that *Polygala tenuifolia*, and perhaps other plant extracts, contain ligands for receptors that modulate neuronal growth and differentiation also requires further investigation.

Apocynum lancifolium leaf is reported to contain cardiac glycosides (e.g. cymarin (**48**)), flavones (e.g. quercetin (**104**)), flavonol-*O*-glycosides (e.g. hyperin), phenylpropanoid substituted flavan-3-ols (e.g. apocynins A - D), lauric acid, phenolic compounds, steroid saponins, coumarins (e.g. scopoletin) and triterpenes (e.g. α-amyrin and lupeol) (Chang and But, 1987; Duke and Ayensu, 1995; Fan *et al.*, 1999; Huang, 1993; Jianming, 1988; Miaohua and Fengshan, 1991). Amino acids including GABA (**16**) were also detected in the leaf extracts (refer to Chapter 2, 2.2.6).

Z. jujuba var. *spinosa* seeds are reported to contain saponins (e.g. jujubosides A and B), flavone-*C*-glycosides (e.g. swertisin (**150**) and spinosin (**149**)), triterpenes (e.g. betulic acid and betulin), and fixed oil (Chang and But, 1987; Tang and Eisenbrand, 1992). Some amino acids, but not GABA, were also detected in the seed extracts (refer to Chapter 2, 2.2.6).

Apocynum lancifolium leaf and *Z. jujuba* var. *spinosa* seeds, nor their known constituents, have previously been investigated for their effects on NSC differentiation. Of the phytochemicals previously investigated for effects on neuronal differentiation, euxanthone (**151**), a xanthone isolated from *Polygala caudata* root, promoted neuronal growth and differentiation, identified using a marker for MAP-2, in neuroblastoma (Neuro-2A) cells, which is a neuropeptide-producing cell line (Mak *et al.*, 2000).



Euxanthone (**151**)

Polygala tenuifolia root (a plant also used in the present study) extract, but not *Z. jujuba* var. *spinosa* seed extract, significantly increased NGF secretion in astroglial cells *in vitro* (Yabe *et al.*, 1997). *Polygala tenuifolia* root is also reported to contain xanthones (Fujita *et al.*, 1992; Huang, 1993; Ikeya *et al.*, 1991a; Ikeya *et al.*, 1994;

Sakuma and Shoji, 1981a; Sakuma and Shoji, 1981b; Tang and Eisenbrand, 1992). Xanthones may therefore explain the reported effects of *Polygala caudata* and *Polygala tenuifolia*, and if present in the active extracts used in the present study, may explain their effects on NSC differentiation. The lack of notable effects of *Polygala tenuifolia* on NSC differentiation in the present study may reflect the different cell types used for analysis, or variation in the chemical composition of the *Polygala tenuifolia* roots used in each study.

A MeOH extract of *W. somnifera* root dose-dependently promoted dendrite extension in neuroblastoma (SK-N-SH) cells *in vitro* (Tohda *et al.*, 2000). The cytotoxicity observed with *W. somnifera* root EtOH extract may explain why no effects on NSC differentiation were observed in the present study. It cannot be excluded that a MeOH extract of this herb may be less cytotoxic and could possibly potentiate NSC differentiation, but this requires investigation.

6.3.3 Conclusion

Grafts of foetal basal forebrain tissue into brain in animal models of AD and lesion-induced memory impairment, have been reported to enhance cognitive function (Fine *et al.*, 1985; Welner *et al.*, 1988). Factors that promote the development of a specific neuronal population and support neuronal survival *in vitro* could be important in the success of neuronal cell transplantation. For example, NSCs treated with FGF-2 *in vitro*, promoted long-term neuronal survival following transplantation into basal forebrain *in vivo* (Minger *et al.*, 1996).

It is apparent that both the aqueous and EtOH extracts of *Apocynum lancifolium* leaf and *Z. jujuba* var. *spinosa* seed promote neuronal differentiation *in vitro* (Figures 6.6 - 6.9 and 6.11 - 6.12). Although these results are preliminary, these plant extracts may be a source of compounds with particular relevance in the treatment of neurodegenerative diseases. The extensive effects on neurite outgrowth observed with these plant extracts *in vitro*, indicate that administration of the active compounds *in vivo* (either to influence existing neurons *in vivo*, or to promote neuronal differentiation following NSC graft transplantation) may have potential in the treatment of paralysis caused by spinal cord transection. If further studies confirm that the active plant extracts promote the development of a specific neuronal

phenotype such as cholinergic or dopaminergic neurons, this may be particularly relevant in the treatment of AD and Parkinson's disease respectively.

In conclusion, further studies are clearly warranted to identify the active compounds present in the plant extracts and their specific effects on neuronal differentiation. It may be that numerous compounds, each specific for the promotion of a particular neuronal phenotype, may be isolated. Isolated compounds may also act as templates for the development of more active or specific compounds that modify neuronal differentiation. The active compounds isolated from the plant extracts may also have advantages over other compounds that influence neuronal differentiation (e.g. NGF), in that they may cross the BBB to promote neurite outgrowth in the CNS.

CHAPTER 7

General Discussion and Conclusions

The aim of the investigations in this study was to identify a pharmacological basis for plants with reputed anti-aging or memory-enhancing effects used in traditional Ayurvedic, Chinese and European medicine. Most of the plants showed pharmacological activities that may explain their use in traditional medicine, and subsequently they may have potential in the treatment of cognitive and neurodegenerative disorders such as AD. However, it must also be considered that other pharmacological activities that were not investigated in the present study may also explain the reputed activities of the plants, and further investigations would provide even greater understanding of their pharmacological activities. The plant extracts could also be investigated for other activities that may be relevant in AD therapy, for example muscarinic or nicotinic receptor binding, or effects on β-amyloid deposition. As knowledge of the pathological mechanisms that occur in AD becomes clearer, the approaches to treatment may differ from current therapy. The plants with reputed effects on cognition could also be assessed for other effects on the pathological mechanisms that occur in AD as they become more fully understood.

Fractionation of the active plant extracts is necessary to identify which compounds are responsible for the pharmacological activities observed, or if compounds act synergistically to enhance activity. It must also be considered that the majority of investigations in the present study were *in vitro* bioassays, so although the results from these studies may show extracts and essential oils to have relevant activities in relation to AD, further testing *in vivo* is necessary to establish their therapeutic potential. *In vivo* pharmacokinetic studies would be necessary to assess the absorption, metabolism and elimination of extracts and compounds, whilst access of active compounds across the BBB and toxicity should also be evaluated. Extracts that showed activity *in vitro* may not necessarily be active *in vivo*, perhaps to due metabolic conversion to inactive compounds. Similarly, extracts that were inactive *in vitro* could show activity *in vivo*, perhaps because of metabolic conversion to active compounds.

In the present study, all of the plants investigated except two (*Codonopsis pilulosa* root and *Gentiana* spp. (found substituted for *Polygonum multiflorum* root), refer to Chapter 2, 2.2.1.1), showed at least one pharmacological activity that may be relevant in AD therapy. This suggests that selection of plants with regard to their ethnobotanical uses is a relatively successful approach to identify pharmacological activities that may be relevant to a particular disorder. The main conclusions regarding the pharmacological activities of each plant in this study, and their relevance in AD therapy, are discussed below.

7.1 Plants Investigated in this Study

7.1.1 *Alisma orientalis*

The cold ethanolic extract of *Alisma orientalis* root significantly inhibited lipid peroxidation *in vitro* (refer to Chapter 6, 6.1.2.2). This extract may therefore be useful in disorders where oxidative processes may be contributing factors, such as carcinogenesis and AD. The compounds responsible for anti-oxidant activity and their potency require further investigation. As discussed previously (refer to 6.1.3), the extract (and active compounds) also requires evaluation in other anti-oxidant systems *in vitro* and *in vivo* before the characterisation of compounds as anti-oxidants. *A. orientalis* root extracts did not inhibit AChE activity *in vitro* (refer to Chapter 3, 3.2.2), did not show significant oestrogenic activity *in vitro* (refer to Chapter 4, 4.5.1), were not significantly active against eicosanoid formation *in vitro* (refer to Chapter 5, 5.2.1) and the ethanolic extract was cytotoxic to neural stem cells (NSCs) *in vitro*, so did not significantly influence NSC differentiation (refer to Chapter 6, 6.3.2). Any favourable effects of *A. orientalis* root on cognitive and age-related disorders therefore, is unlikely to be due to anti-inflammatory mechanisms, oestrogenic activity or inhibition of AChE, but may be due to other pharmacological activities, perhaps anti-oxidant activity.

7.1.2 *Apocynum lancifolium*

Both the aqueous and ethanolic extracts of *Apocynum lancifolium* leaf significantly inhibited AChE activity *in vitro*, with the aqueous extract being the more active (refer to Chapter 3, 3.2.2). Fractionation of these extracts is necessary to identify which compounds were responsible for this activity. As the aqueous extract was more active than the ethanolic extract, the most active compounds may be more polar, and are perhaps the cardiac glycosides. Cymarin (**48**), a constituent of *A. lancifolium* leaf, only weakly inhibited AChE (refer to Chapter 3, 3.2.4.2) but it cannot be excluded that other cardiac glycosides present in the extracts were more potent inhibitors of AChE.

Both the aqueous and ethanolic extracts of *A. lancifolium* leaf influenced NSC differentiation *in vitro* (refer to Chapter 6, 6.3.2). Compounds isolated from these extracts may therefore have potential in the treatment of neurodegenerative disorders, including AD, Parkinson's disease and spinal cord injury. Further investigations are necessary to identify active compounds and establish their therapeutic potential *in vivo*.

Neither the aqueous nor the ethanolic extract of *A. lancifolium* leaf showed significant oestrogenic activity *in vitro* (refer to Chapter 4, 4.5.1), nor were they significantly active against eicosanoid formation *in vitro* (refer to Chapter 5, 5.2.1).

The reputed effects of this plant on cognitive disorders may be explained by enhancement of cholinergic function via inhibition of AChE, but this requires confirmation *in vivo*. This plant may potentially yield new (and perhaps novel, if it is confirmed to be the cardenolide compounds that are antiChEs) AChE inhibitors that may be used for the symptomatic treatment of AD.

7.1.3 *Centella asiatica*

All extracts of *Centella asiatica* leaf inhibited AChE activity *in vitro*, in the order of potency: aqueous extract < ethanolic extract < petroleum spirit extract (refer to Chapter 3, 3.2.2) and, the ethanolic extract of *Centella asiatica* leaf was significantly active against TXB_2 formation *in vitro* (refer to Chapter 5, 5.2.1). The hot ethanolic extract of *C. asiatica* leaf significantly inhibited lipid peroxidation *in vitro* (refer to Chapter 6, 6.1.2.2). None of the *C. asiatica* leaf extracts showed significant

oestrogenic activity *in vitro* (refer to Chapter 4, 4.5.1), and the ethanolic extract was cytotoxic to NSCs *in vitro* and so did not significantly influence NSC differentiation (refer to Chapter 6, 6.3.2).

The reputed effects of this plant on memory may therefore be due to a combination of activities, including antiChE, anti-inflammatory and anti-oxidant effects. The ethanolic extract showed the greatest variety of pharmacological activities, compared to the aqueous and petroleum spirit extracts. It remains to be investigated if the same compounds were responsible for all the pharmacological activities observed, or if different compounds were responsible for each activity. If the latter is confirmed by fractionation and isolation, use of the crude extract that has multiple activities may be more appropriate than the use of isolated compounds, which may only have one beneficial effect in relation to AD treatment. The results from the *in vitro* studies show that *C. asiatica* leaf may also be a source of new therapeutic agents that are not only appropriate for use in AD, but may also provide compounds or template compounds for other disorders, including inflammatory diseases such as rheumatoid arthritis.

7.1.4 *Codonopsis pilulosa*

Codonopsis pilulosa root extracts did not inhibit AChE activity *in vitro* (refer to Chapter 3, 3.2.2), did not show significant oestrogenic activity *in vitro* (refer to Chapter 4, 4.5.1), were not significantly active against eicosanoid formation *in vitro* (refer to Chapter 5, 5.2.1), the hot ethanolic extract did not inhibit lipid peroxidation *in vitro* (refer to Chapter 6, 6.1.2.2) and although not cytotoxic, the ethanolic extract did not significantly influence NSC differentiation *in vitro* (refer to Chapter 6, 6.3.2). These results indicate that the reputed anti-amnesic effect of *C. pilulosa* root in TCM may not be due to inhibition of AChE, inhibition of eicosanoid generation, inhibition of lipid peroxidation or oestrogenic activity. Further studies are necessary to identify any other pharmacological basis for the reputed effects of this plant.

Although no pharmacological activities were identified with this plant in the present study, the root may have other pharmacological activities relevant to AD treatment. For example, the root extracts may have activities *in vivo* that do not occur *in vitro*, perhaps due to metabolic conversion to active compounds *in vivo*.

7.1.5. *Convallaria majalis*

The dichloromethane and ethanolic extracts (not the aqueous or the hexane extracts) of *Convallaria majalis* leaf significantly inhibited AChE activity *in vitro* (refer to Chapter 3, 3.2.4.1). Several fractions of the ethanolic extract and also the dichloromethane fraction of the ethanolic extract of *C. majalis* leaf, obtained by flash column chromatography (FCC) significantly inhibited AChE activity *in vitro* (refer to Chapter 3, 3.2.4.2 and 3.2.4.3). The active fractions obtained by FCC were analysed using TLC to identify the types of compounds that may have been responsible for activity. Neither flavonoids nor alkaloids were detected in active fractions obtained (FCC (a)), but two fractions (F3a and F8a) each contained a different unidentified cardenolide, indicating that cardenolides may have contributed to the antiChE activity of these active fractions (refer to Chapter 2, 2.2.2.1 and 2.2.2.5, and Chapter 3, 3.2.4.2).

The commercially obtained cardenolides convallatoxin (**50**) and cymarin (**48**) (which are found in *C. majalis* and *Apocynum lancifolium* respectively) showed no, or only weak, activity against AChE activity respectively (refer to Chapter 3, 3.4.2). Cardenolide compounds were not detected in other fractions obtained from FCC (a), which suggests that compounds other than cardenolides contributed to the antiChE activity of these fractions (refer to Chapter 2, 2.2.2.1).

Fractionation of the ethanolic extract of *C. majalis* leaf using droplet counter-current chromatography (DCCC) and preparative thin-layer chromatography (TLC), also yielded several fractions that significantly inhibited AChE activity *in vitro* (refer to Chapter 3, 3.2.4.4 and 3.2.4.5). The most active fraction (F4) obtained using DCCC did not contain constituents that were common to the active fractions F8a from FCC (a) and F3 and F4 from preparative TLC separation (refer to Chapter 2, 2.2.2.4), confirming that different compounds were responsible for antiChE activity of the active fractions, and that they may have acted synergistically in the crude extract.

The compounds present in the preparative TLC fractions were investigated using TLC and LC-MS, and compounds present in the active fractions F3 and F4 were shown not to be alkaloids, flavonoids or chlorophyll, and using TLC analysis, three zones in F3 and F4 were common to F8a from FCC (a), indicating that the compounds in these zones may have been responsible for the antiChE activity observed (refer to Chapter

2, 2.2.2.5 and 2.2.3.2, and Chapter 3, 3.2.4.2 and 3.2.4.5). HPLC analysis also confirmed the absence of flavonoid compounds in F3 (refer to Chapter 2, 2.2.3.2).

It may be concluded that *C. majalis* leaf is a potential source of new AChE inhibitors, which are not flavonoids or alkaloids but may be other unidentified compounds, perhaps cardenolides. Further fractionation of the ethanolic and dichloromethane extracts is required to confirm which compounds were responsible for the antiChE activity, and their potency *in vivo*. Isolation of the antiChE compounds may lead to the development of novel AChE inhibitors for AD therapy, particularly if confirmed not to be the known types of AChE inhibitor i.e. the alkaloids and monoterpenes.

The ethanolic extract of *C. majalis* leaf was also significantly active against TXB_2 formation *in vitro* (refer to Chapter 5, 5.2.1). This result indicates that the leaf extract may have anti-inflammatory activity via inhibition of COX, but further fractionation and isolation is necessary to identify the compounds responsible for this activity. None of the *C. majalis* leaf extracts showed significant oestrogenic activity *in vitro* (refer to Chapter 4, 4.5.1) and the ethanolic extract was cytotoxic to NSCs *in vitro*, so did not influence NSC differentiation (refer to Chapter 6, 6.3.2).

The claims in European traditional medicine that *C. majalis* leaf may enhance memory could perhaps be explained by enhancement of cholinergic function via inhibition of AChE and perhaps anti-inflammatory activity. It would also be important to investigate whether the same compounds are responsible for both activities. Confirmation of this may provide an AChE inhibitor with additional anti-inflammatory activity, which may have advantages over existing AChE inhibitors used for symptomatic treatment of AD.

7.1.6 *Gentiana* spp. (Adulterated *Polygonum multiflorum*)

The original aim of this study was to investigate if *Polygonum multiflorum* root, which is reputed in TCM to restore youth and treat neurosis, showed pharmacological activities that may be useful in AD treatment. However, authentication of the root used in the bioassays in the present study, showed that it was not *P. multiflorum* but a species of *Gentiana* (refer to Chapter 2, 2.2.1.1). Therefore the pharmacological basis for the reputed effects of *P. multiflorum* root still requires further investigation.

Gentiana spp. root (adulterated *P. multiflorum* root) extracts did not inhibit AChE activity *in vitro* (refer to Chapter 3, 3.2.2), nor did they show significant oestrogenic

activity *in vitro* (refer to Chapter 4, 4.5.1) and were not significantly active against eicosanoid formation *in vitro* (refer to Chapter 5, 5.2.1); neither the hot nor the cold ethanolic extracts significantly inhibited lipid peroxidation *in vitro* (refer to Chapter 6, 6.1.2.2). Although not cytotoxic, the ethanolic extract also showed no significant effect on NSC differentiation *in vitro* (refer to Chapter 6, 6.3.2). These results show that a *Gentiana* spp., a plant that does not have a reputation for enhancing memory or anti-aging effects, does not appear to have pharmacological activities that may alleviate such cognitive disorders.

7.1.7 *Melissa officinalis*

The *Melissa officinalis* leaf extracts, essential oils and oil constituents showed numerous pharmacological effects that may be appropriate in AD therapy. Both the *M. officinalis* leaf aqueous extract and the essential oil significantly inhibited AChE activity *in vitro* (refer to Chapter 3, 3.2.2). This result indicates that both polar (from the aqueous extract) and less polar compounds (present in the essential oil) from *M. officinalis* were antiChE. The compounds responsible for the antiChE activity of the aqueous extract require further investigation by fractionation and isolation of the active compounds; the antiChE activity of the essential oil may be due to the monoterpenes citral, geraniol (**72**) and linalool (**74**) (these compounds were identified in *M. officinalis* essential oil using GC-MS analysis; refer to Chapter 2, 2.2.7.1), as these monoterpenes have previously been reported to inhibit AChE (Ryan and Byrne, 1988; Perry *et al.*, 2000a).

Neither the aqueous nor the ethanolic extracts of *M. officinalis* leaf showed significant oestrogenic activity *in vitro* (refer to Chapter 4, 4.5.1). The *M. officinalis* essential oil, phytol and crude phytol extracts, and the essential oil constituents (citral, citronellol (**69**), geraniol (**72**), nerol (**75**), nerolidol (**83**), *trans*-caryophyllene (**82**) and nonanal (**86**)) showed significant dose-dependent oestrogenic activity in genetically modified yeast cells *in vitro*, but neither the essential oil nor the monoterpenes citral and geraniol (**72**), showed significant oestrogenic activity in human Ishikawa cells *in vitro* (refer to Chapter 4, 4.6 and 4.7). They also showed no oestrogenic activity *in vivo* following transdermal administration to mice (refer to Chapter 4, 4.9). Citral, eugenol (**84**), geraniol (**72**) and nerol (**75**) did however, dose-dependently displace

[^{3}H]-17β-oestradiol binding to ERα and ERβ *in vitro* (refer to Chapter 4, 4.8.2) and eugenol (**84**) showed weak ER antagonistic activity *in vitro* (refer to Chapter 4, 4.10). Although these results, regarding the oestrogenic activity of the monoterpenes, are not conclusive, it is clear that there is potential for oestrogenic activity to occur *in vivo* perhaps following longer-term exposure. This may be relevant in AD as oestrogenic compounds have been proposed to protect against AD, and ERα and ERβ have been detected in the amygdala, hippocampus and cerebral cortex (Butler *et al.*, 1999; Osterlund *et al.*, 1998; Osterlund *et al.*, 2000; Register *et al.*, 1998). The monoterpenes are relatively low molecular-weight lipophilic molecules; criteria that may permit their access across the BBB (Pardridge, 1998). These monoterpenes may therefore interact with ERα and ERβ in the CNS and influence cognitive function, or they may act as templates for the development of other oestrogenic compounds. The adverse effects of human (and other animal) exposure to potentially oestrogenic monoterpenes must be given careful consideration, as cumulative exposure may promote carcinogenesis, reproductive disorders and emasculating effects in males. The anti-oestrogenic activity of eugenol (**84**) could be exploited for the development of compounds for the treatment of hormone-dependent cancers.

The ethanol extract and the essential oil of *M. officinalis* leaf and the oil constituents citral, geraniol (**72**) and nerol (**75**) demonstrated dose-dependent activity against TXB_2 and LTB_4 formation *in vitro*; *trans*-caryophyllene (**82**) also showed activity against LTB_4 formation *in vitro* (refer to Chapter 5, 5.2.3). These results indicate that the less polar compounds, including monoterpenes, from *M. officinalis* may have anti-inflammatory activity. Further fractionation and isolation of the active compounds in the ethanolic extract are required.

The aqueous extract, but not the ethanolic extract, of *M. officinalis* leaf significantly displaced [^{3}H]-GABA binding to $GABA_B$ and $GABA_A$ receptors *in vitro* (refer to Chapter 6, 6.2.2.1 and 6.2.2.2). This extract was not highly selective for either receptor, which may be inappropriate in clinical use as the potential for adverse effects may be greater. However, the essential oil of *M. officinalis* and the oil constituents citral, geraniol (**72**) and nerol (**75**) displaced [^{3}H]-GABA binding to $GABA_B$ receptors more selectively than displacement of binding to $GABA_A$ receptors *in vitro* (refer to Chapter 6, 6.2.2.1 and 6.2.2.2). It is therefore apparent that polar compounds from the aqueous extract, which require identification, and less polar monoterpenes from the essential oil may interact with GABA receptors and perhaps

influence cognition. The active monoterpenes identified in the essential oil were more selective for $GABA_B$ receptors than $GABA_A$ receptors, which may be associated with fewer adverse effects. It is unknown if the active compounds would induce an agonistic or antagonistic response *in vivo*. It requires further investigation to determine if antagonistic binding occurs at the $GABA_B$ receptor, an effect that may promote favourable effects in cognitive function. Agonistic activity at the $GABA_B$ receptor may be more appropriate for the treatment of spasticity.

The differences in the pharmacological activity and potency between each extract (aqueous and ethanolic extracts) and the essential oil, illustrate how the diverse mixture of compounds in a plant may result in various pharmacological effects, and that compounds may act synergistically. It is also apparent that the monoterpenes may have numerous activities, including oestrogenic, anti-inflammatory or GABA receptor binding effects. This may reflect their general lipophilicity, which may permit favourable hydrophobic interactions with receptors and enzymes. The precise nature of these interactions requires further study, but the molecular graphics investigations in the present study give some indication of the ligand-binding of the monoterpenes and eugenol (**84**) in ERα (refer to Chapter 4, 4.11).

The *M. officinalis* essential oil did not significantly inhibit lipid peroxidation *in vitro* (refer to Chapter 6, 6.1.2.2), and the ethanolic extract of *M. officinalis* leaf was cytotoxic to NSCs *in vitro*, so did not influence NSC differentiation (refer to Chapter 6, 6.3.2). Oestrogenic, anti-inflammatory and GABA receptor binding activities may therefore explain the reputed memory-enhancing effects of *M. officinalis* in traditional European medicine.

7.1.8 *Polygala tenuifolia*

The ethanolic extract of *Polygala tenuifolia* root showed significant oestrogenic activity in both genetically modified yeast cells and human Ishikawa cells *in vitro* (refer to Chapter 4, 4.5.1 and 4.5.2). The compounds responsible for the apparent oestrogenic effects require isolation, and their activity confirmed in receptor binding studies and *in vivo*.

The cold ethanolic extract and the hot aqueous extract of *P. tenuifolia* root significantly inhibited lipid peroxidation *in vitro* (refer to Chapter 6, 6.1.2.2). This suggests that both polar and less polar compounds present in the root were able to

inhibit lipid peroxidation. Oestrogens are reported to have anti-oxidant activity (Behl *et al.*, 1995; Mooradian, 1993; Ruiz-Larrea *et al.*, 1994). Thus, it is possible that the oestrogenic compounds in the ethanolic extract were also responsible for the anti-oxidant effects observed. Confirmation of the anti-oxidant and oestrogenic effects *in vivo* is required.

P. tenuifolia root extracts did not inhibit AChE activity *in vitro* (refer to Chapter 3, 3.2.2); nor did they significantly inhibit eicosanoid formation *in vitro* (refer to Chapter 5, 5.2.1). The ethanolic extract was cytotoxic to NSCs *in vitro* and did not influence NSC differentiation (refer to Chapter 6, 6.3.2). The proposed anti-amnesic effects of *P. tenuifolia* root are perhaps not due to antiChE or anti-inflammatory effects, but may be due to a combination of oestrogenic and anti-oxidant effects.

7.1.9 *Rosmarinus officinalis*

Both the aqueous and ethanolic extracts of *Rosmarinus officinalis* dried leaf and the essential oil, significantly inhibited AChE activity *in vitro*; the ethanolic extract of the fresh leaf did not significantly inhibit AChE activity *in vitro* (refer to Chapter 3, 3.2.2). The antiChE compounds present in the aqueous and ethanolic extracts of the dried leaf are unknown until fractionation and isolation of the extracts is conducted. The compounds responsible for the antiChE activity of the essential oil may be 1, 8-cineole (**56**), camphor (**57**) and limonene (**73**) as these known constituents of *R. officinalis* essential oil have previously been reported to inhibit AChE (Grundy and Still, 1985; Perry *et al.*, 2000a)

The ethanolic extract of *R. officinalis* dried leaf was significantly active against TXB_2 and LTB_4 formation *in vitro*; the ethanolic extract of the fresh leaf was significantly active against LTB_4 formation *in vitro* (refer to Chapter 5, 5.2.1). Further analysis of the active compounds is necessary to identify any compounds that may have potential in the treatment of inflammatory disorders, including AD.

Neither the essential oil, nor the extracts of the fresh leaf or the dried leaf of *R. officinalis* showed significant oestrogenic activity *in vitro* (refer to Chapter 4, 4.5.1 and 4.6.1) and, the ethanolic extracts of the dried and fresh leaf and the essential oil of *R. officinalis* did not significantly displace [^{3}H]-GABA binding to $GABA_B$ or $GABA_A$ receptors *in vitro* (refer to Chapter 6, 6.2.2.1 and 6.2.2.2). The ethanolic extract of the

dried leaf was cytotoxic to NSCs *in vitro*, and no effect on NSC differentiation was observed (refer to Chapter 6, 6.3.2).

It may be concluded that the antiChE and anti-inflammatory effects of *R. officinalis* leaf may explain the reputed favourable effects of this herb in traditional European medicine, and *R. officinalis* may be a source of new antiChE and anti-inflammatory compounds for use in future AD therapy.

7.1.10 *Salvia miltiorrhiza*

Both the aqueous and ethanolic extracts of *Salvia miltiorrhiza* root dose-dependently inhibited AChE activity *in vitro*, with the ethanolic extract being the most active (refer to Chapter 3, 3.2.3). The ethanolic extract of *S. miltiorrhiza* root also showed significant activity against TXB_2 and LTB_4 formation but the less active aqueous extract showed weaker activity against TXB_2 formation *in vitro* (refer to Chapter 5, 5.2.1.3). The hot aqueous extract and the cold and hot ethanolic extracts of *S. miltiorrhiza* root significantly inhibited lipid peroxidation *in vitro* (refer to Chapter 6, 6.1.2.2).

The compounds present in the root that contributed to these activities also need further assessment. Tanshinone I (**115**), dihydrotanshinone, tanshinone IIa (**116**) and cryptotanshinone (**117**) isolated from the lipophilic extracts of *S. miltiorrhiza* root have previously shown activity against 5-LOX formation in porcine leukocytes (Paulus and Bauer, 2000). These diterpenes may have also been responsible for the inhibition of eicosanoid generation in the present study. Several compounds present in the root have also shown anti-oxidant effects. These include the water-soluble salvianolic acids A (**123**) and B (**124**) (Huang and Zhang, 1992; Liu *et al.*, 1992) and several quinones, including dehydrorosmariquinone, rosmariquinone, miltirone I, tanshinone I (**115**), cryptotanshinone (**117**), dihydrotanshinone and methylenetanshinquinone (Weng and Gordon, 1992; Zhang *et al.*, 1990). The salvianolic acids and the quinones may have been responsible for the anti-oxidant effects of the aqueous and the ethanolic extracts respectively in the present study. Although, the activities may also have been due to other compounds, perhaps by acting synergistically.

S. miltiorrhiza root is therefore also a potential source of antiChE, anti-inflammatory and anti-oxidant compounds. If a particular compound were to be isolated that had

two or more of these pharmacological activities, it might have particular usefulness in AD therapy to maximise efficacy and to reduce dose frequency to patients. Multiple drug therapy may require dosing of numerous drugs daily, which may be problematic in patients with cognitive dysfunction, and administration of a compound with multiple activities, may overcome this problem.

The antiChE, anti-inflammatory and anti-oxidant effects of *S. miltiorrhiza* root may explain its use in TCM. None of the *S. miltiorrhiza* root extracts showed significant oestrogenic activity *in vitro* (refer to Chapter 4, 4.5.1) and the ethanolic extract was cytotoxic to NSCs *in vitro*, and did not influence NSC differentiation (refer to Chapter 6, 6.3.2).

7.1.11 *Withania somnifera*

Both the aqueous and ethanolic extracts of *Withania somnifera* root significantly inhibited AChE activity *in vitro* (refer to Chapter 3, 3.2.2). The ethanolic extract of *W. somnifera* root was significantly active against TXB_2 formation *in vitro* (refer to Chapter 5, 5.2.1) and significantly inhibited lipid peroxidation *in vitro* (refer to Chapter 6, 6.1.2.2). But, none of the *W. somnifera* root extracts showed significant oestrogenic activity *in vitro* (refer to Chapter 4, 4.5.1) and the ethanolic extract was cytotoxic to NSCs *in vitro*, showing no effect on NSC differentiation (refer to Chapter 6, 6.3.2).

Phytochemical analysis of the compounds responsible for these activities is still required. However, as the ethanolic extract showed apparent antiChE, anti-inflammatory (perhaps via inhibition of COX) and anti-oxidant activities, the compounds responsible are likely to be those that are less polar. This plant also has potential for the identification of new therapeutic compounds that may be relevant in AD therapy. Compounds from the root have previously been shown to influence cognitive function. For example, the sitoindosides IX (**65**) and X (**66**) augmented learning acquisition and memory in both young and old rats (Ghosal *et al.*, 1989) and an extract containing the sitoindosides VII - X and withaferin A (**109**) also reversed the ibotenic acid-induced cognitive deficit and reversed the reduction in cholinergic markers (e.g. ACh (**1**), ChAT) in rats (Bhattacharya *et al.*, 1995). The pharmacological activities in the present study may explain these previous

observations, or may provide additional therapeutic activities to those observed previously.

Therefore *W. somnifera* root may have multiple pharmacological activities relevant to the treatment of AD. The use of the crude extract may be advantageous by providing numerous beneficial effects perhaps by compounds acting synergistically, unless compounds were to be isolated that exhibited more than one pharmacological activity.

7.1.12 *Ziziphus jujuba*

The hot aqueous extract of *Ziziphus jujuba* seed significantly inhibited lipid peroxidation *in vitro* (refer to Chapter 6, 6.1.2.2). The seeds are reported to contain flavone *C*-glycosides (e.g. swertisin (**150**), spinosin (**149**)), saponins (e.g. jujubosides A and B) and triterpenoids (e.g. betulin, betulic acid), which may have been responsible for the observed anti-oxidant activity, but their isolation is required to assess their individual potency.

The ethanolic extract of *Z. jujuba* var. *spinosa* seed, showed greater displacement of [^{3}H]-GABA binding to $GABA_B$ receptors, than $GABA_A$ receptors *in vitro*; the aqueous extract did not significantly displace [^{3}H]-GABA binding to $GABA_A$ and $GABA_B$ receptors *in vitro* (refer to Chapter 6, 6.2.2.1 and 6.2.2.2). Selectivity for the $GABA_B$ receptor may be important to minimise adverse effects and to enhance efficacy. Isolation of the active compounds and their potential as $GABA_B$ receptor antagonists *in vivo* is necessary to identify any potential for the use in cognitive disorders, such as AD.

Neither ethanolic extracts of *Z. jujuba* seed or *Z. jujuba* var. *spinosa* seed, nor the aqueous extract of *Z. jujuba* var. *spinosa* seed, were cytotoxic to NSCs *in vitro*, and the aqueous and ethanolic extracts of *Z. jujuba* var. *spinosa* seed consistently showed effects on NSC differentiation (refer to Chapter 6, 6.3.2). These extracts may therefore yield compounds that may support neuronal growth and encourage development of a particular neuronal phenotype (e.g. cholinergic neurons), either by acting directly in the CNS or prior to transplantation of neuronal grafts into brain, for the treatment of neurodegenerative disorders. Investigations in the present study are preliminary, but the promising results warrant the isolation and further investigation of active compounds.

Neither the aqueous nor the ethanolic, nor the petroleum spirit extracts of *Z. jujuba* seed or fruit (or *Z. jujuba* var. *spinosa* seed) inhibited AChE activity *in vitro* (refer to Chapter 3, 3.2.2) and none of the *Z. jujuba*, or *Z. jujuba* var. *spinosa*, seed or fruit extracts showed significant oestrogenic activity *in vitro* (refer to Chapter 4, 4.5.1). Neither the ethanolic nor the aqueous extracts of *Z. jujuba*, or *Z. jujuba* var. *spinosa*, seed were significantly active against eicosanoid formation *in vitro* (refer to Chapter 5, 5.2.1).

The reputed anti-amnesic effects of *Z. jujuba* seeds may be due to anti-oxidant and GABA receptor binding effects, or perhaps effects on neuronal populations, and are perhaps not due to antiChE, oestrogenic or anti-inflammatory effects.

7.2 Conclusion

It is apparent that adulteration of plant material, including substitution of one plant species for another (in the present study a *Gentiana* spp. was substituted for *Polygonum multiflorum*, refer to Chapter 2, 2.2.1.1) may pose numerous problems. Unless the correct species is confirmed, a plant may be reported to have activities (or a lack of activities) that do not reflect the activities of the correct species. Adulterated plant material used clinically may reduce efficacy and perhaps induce toxicity. It is therefore essential that plant material is authenticated prior to use experimentally and clinically.

This study has shown that numerous plant species may be potential sources for compounds that may be relevant in the treatment of various disorders, including cancer (the anti-oxidants and anti-oestrogens), inflammatory disorders such as rheumatoid arthritis (anti-inflammatory compounds) and cognitive disorders such as AD (the anti-oxidants, oestrogenic compounds, anti-inflammatory compounds, antiChE compounds and perhaps GABA receptor ligands and compounds that influence NSC differentiation). The plant extracts that were identified to have the various pharmacological activities in the present study may yield new therapeutic compounds, therefore further work involving the isolation of these active compounds is warranted.

Finally, it must be noted that the majority (>90%) of plants in this study, which were selected on the basis of their reputed cognitive enhancing effects (excluding the

adulterated *Polygonum multiflorum* root), showed at least one pharmacological activity, and many (>70%) showed at least two pharmacological activities, that may be relevant in cognitive disorders. Therefore the pharmacological activities of plants generally appears to reflect their uses in traditional medicine. The ethnobotanical approach for selecting plants to investigate for the treatment of a particular disease, may therefore be very useful in the search for new and effective drugs for a variety of disorders.

APPENDIX

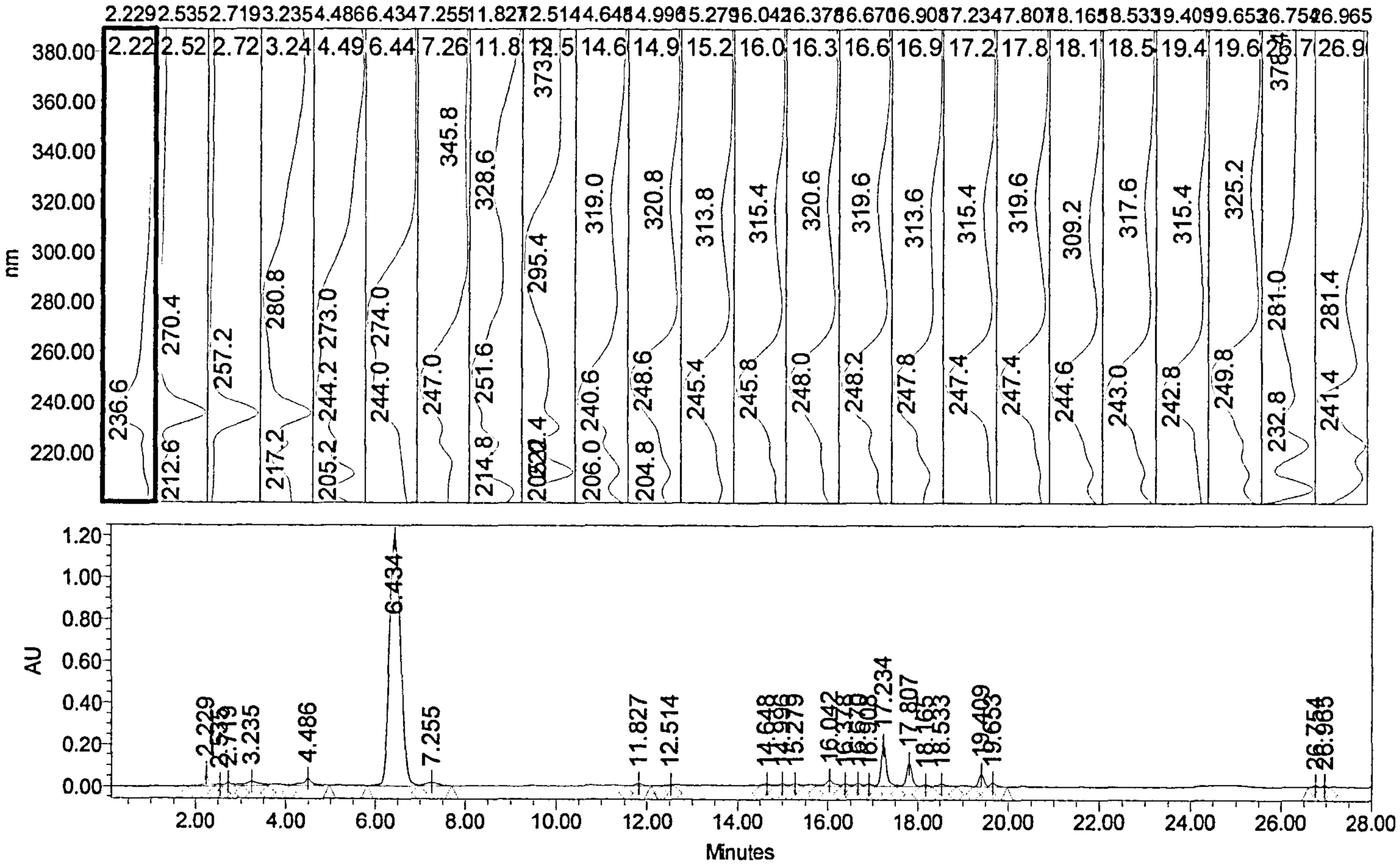


Figure A1. Chromatogram of an acetone extract of a sample claimed to be *Polygonum multiflorum* root, using HPLC analysis and UV detection (210nm).

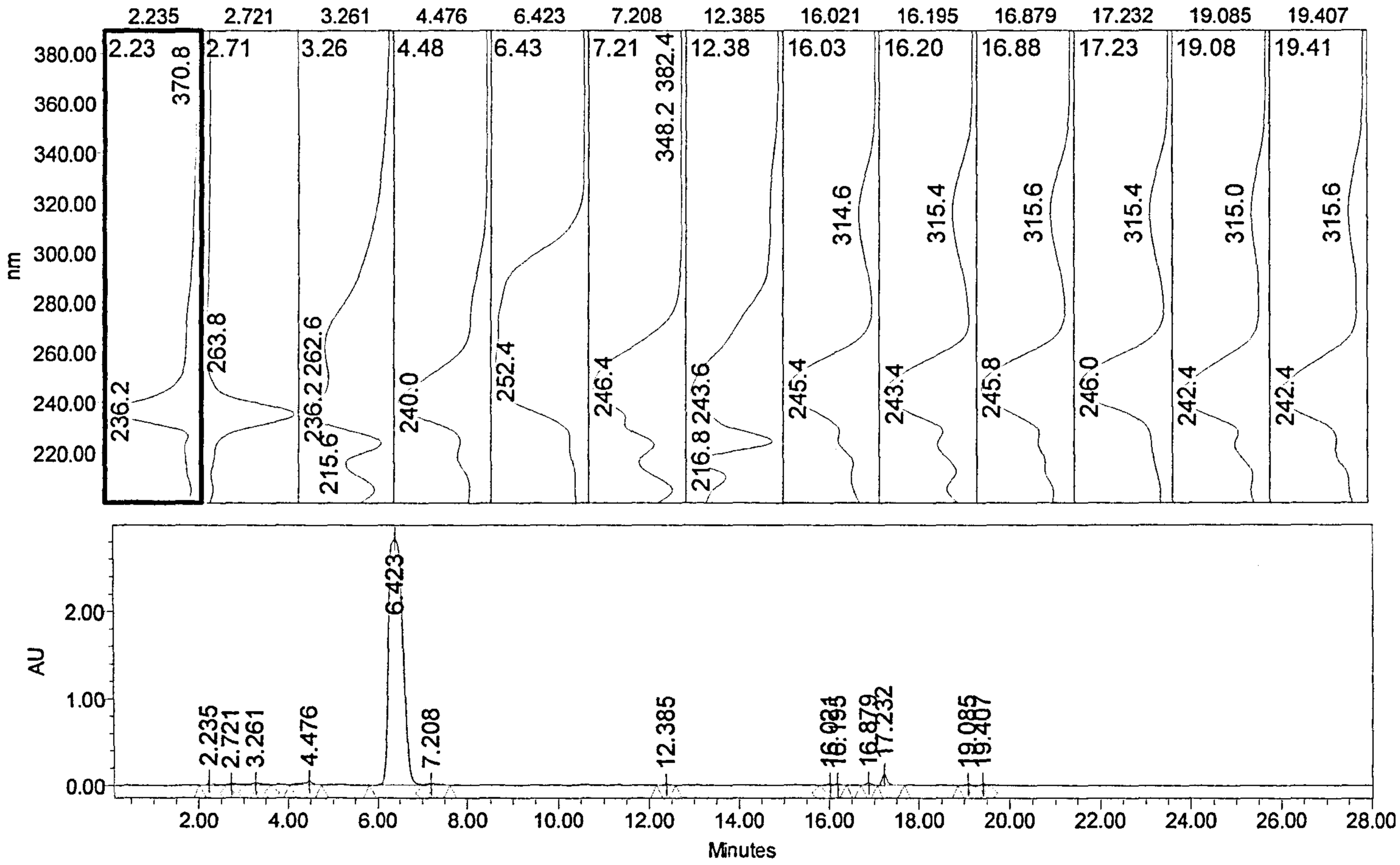


Figure A2. Chromatogram of *Gentiana scabra* root acetone extract using HPLC analysis and UV detection (210nm).

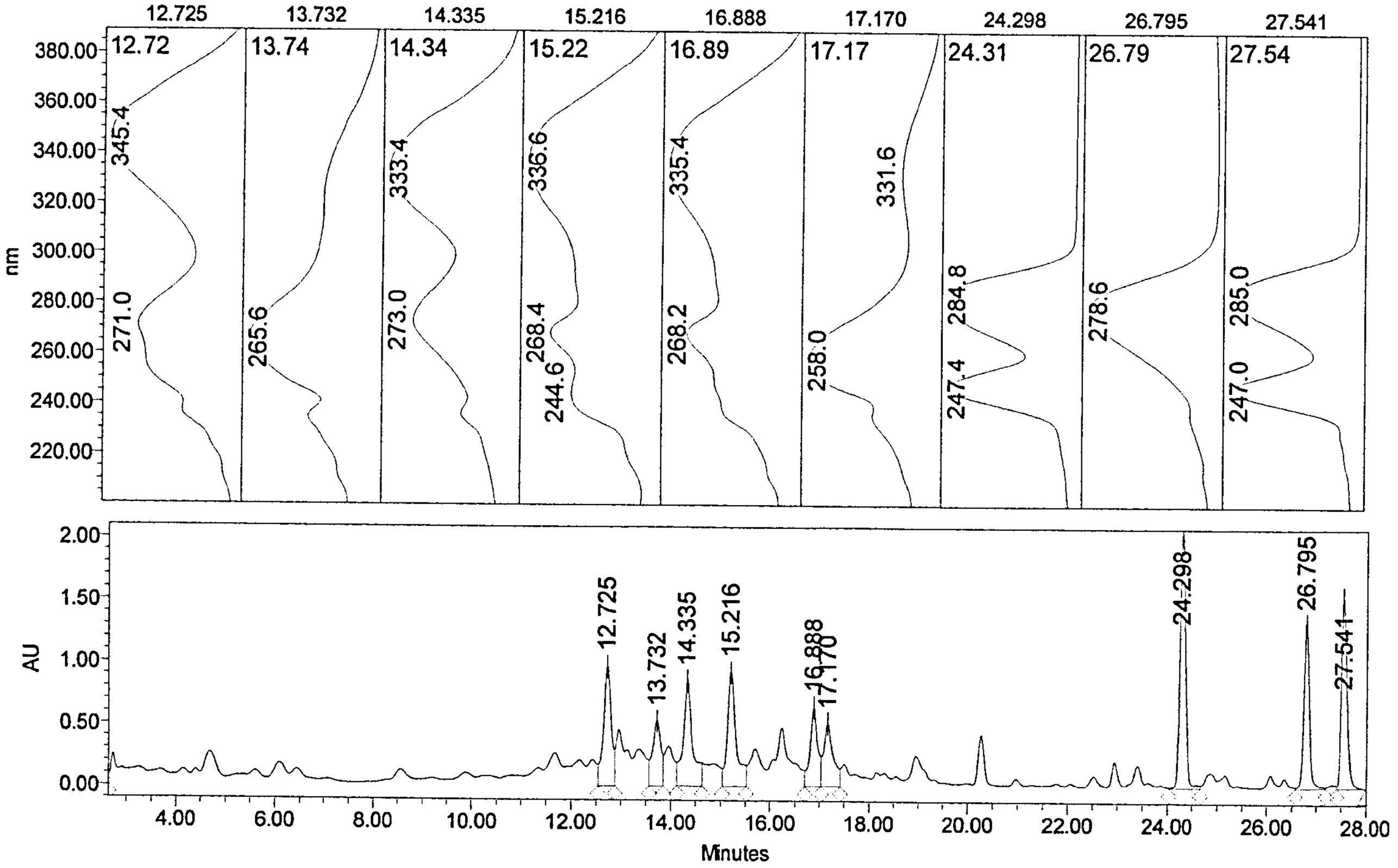


Figure A3. Chromatogram of *Rosmarinus officinalis* fresh leaf (frozen) sample methanol extract using HPLC analysis and UV detection (210nm).

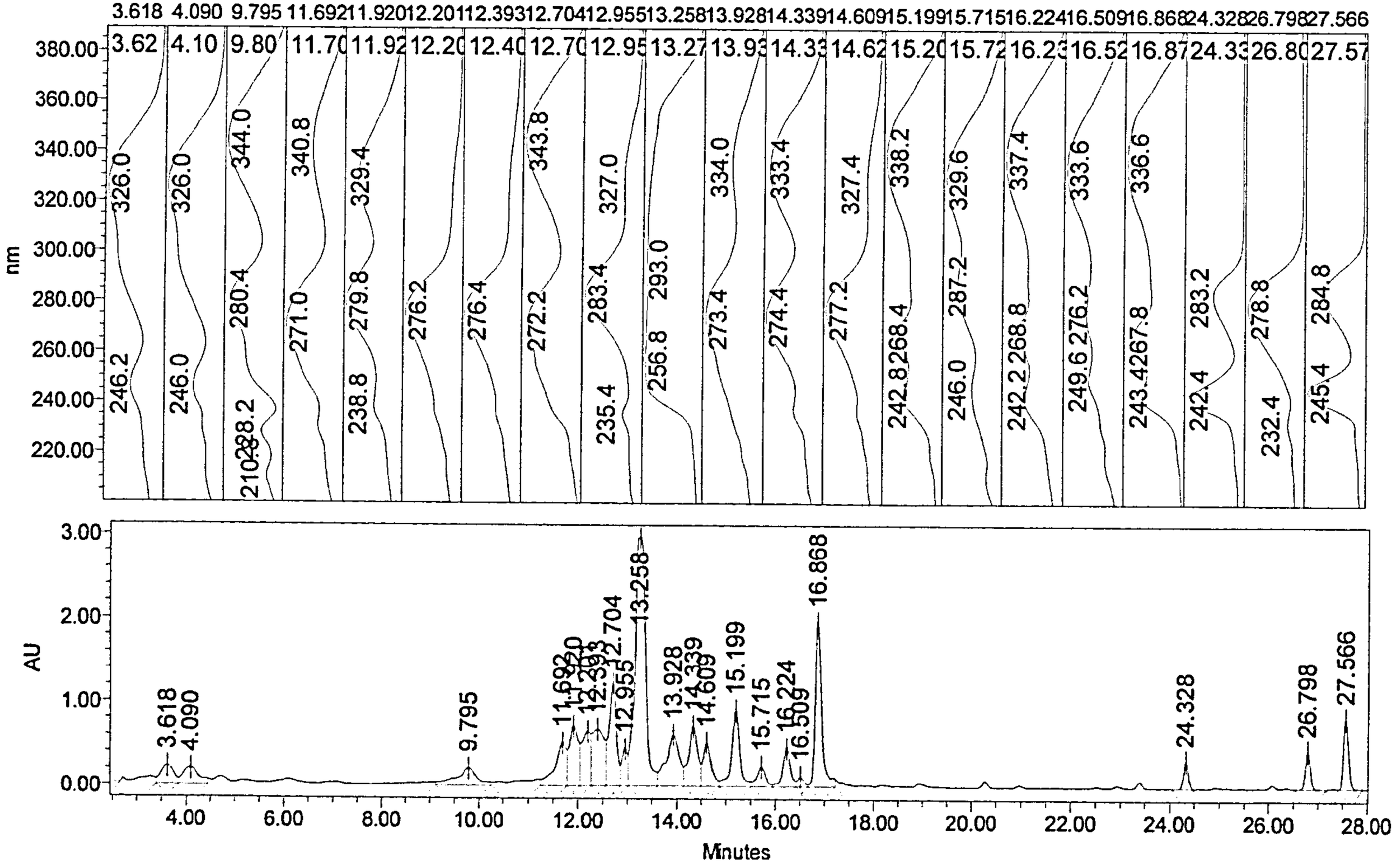


Figure A4. Chromatogram of an authentic sample of *Rosmarinus officinalis* fresh leaf methanol extract using HPLC analysis and UV detection (210nm).

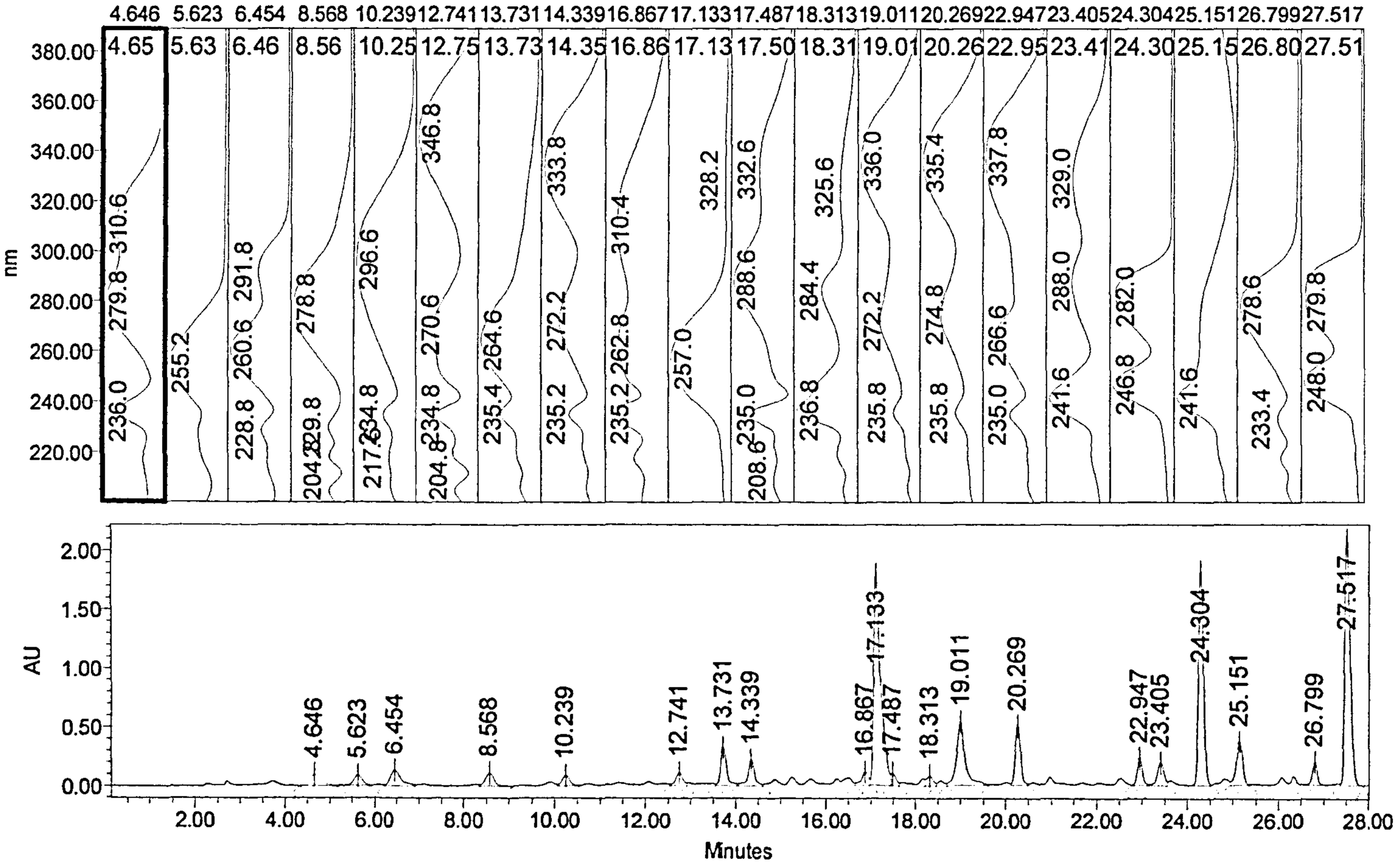


Figure A5. Chromatogram of *Rosmarinus officinalis* fresh leaf (frozen) sample ether extract using HPLC analysis and UV detection (210nm).

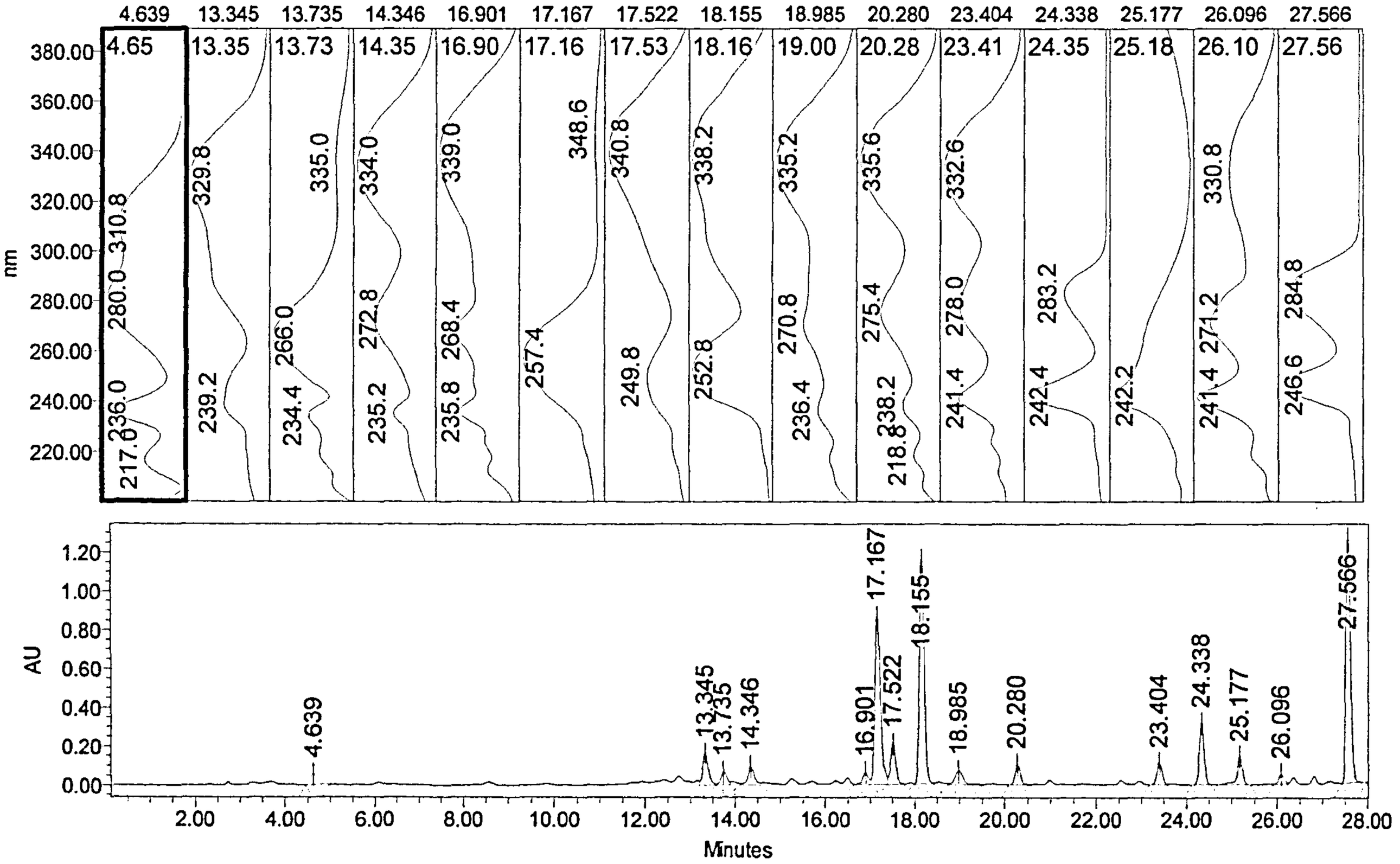


Figure A6. Chromatogram of an authentic sample of *Rosmarinus officinalis* fresh leaf ether extract using HPLC analysis and UV detection (210nm).

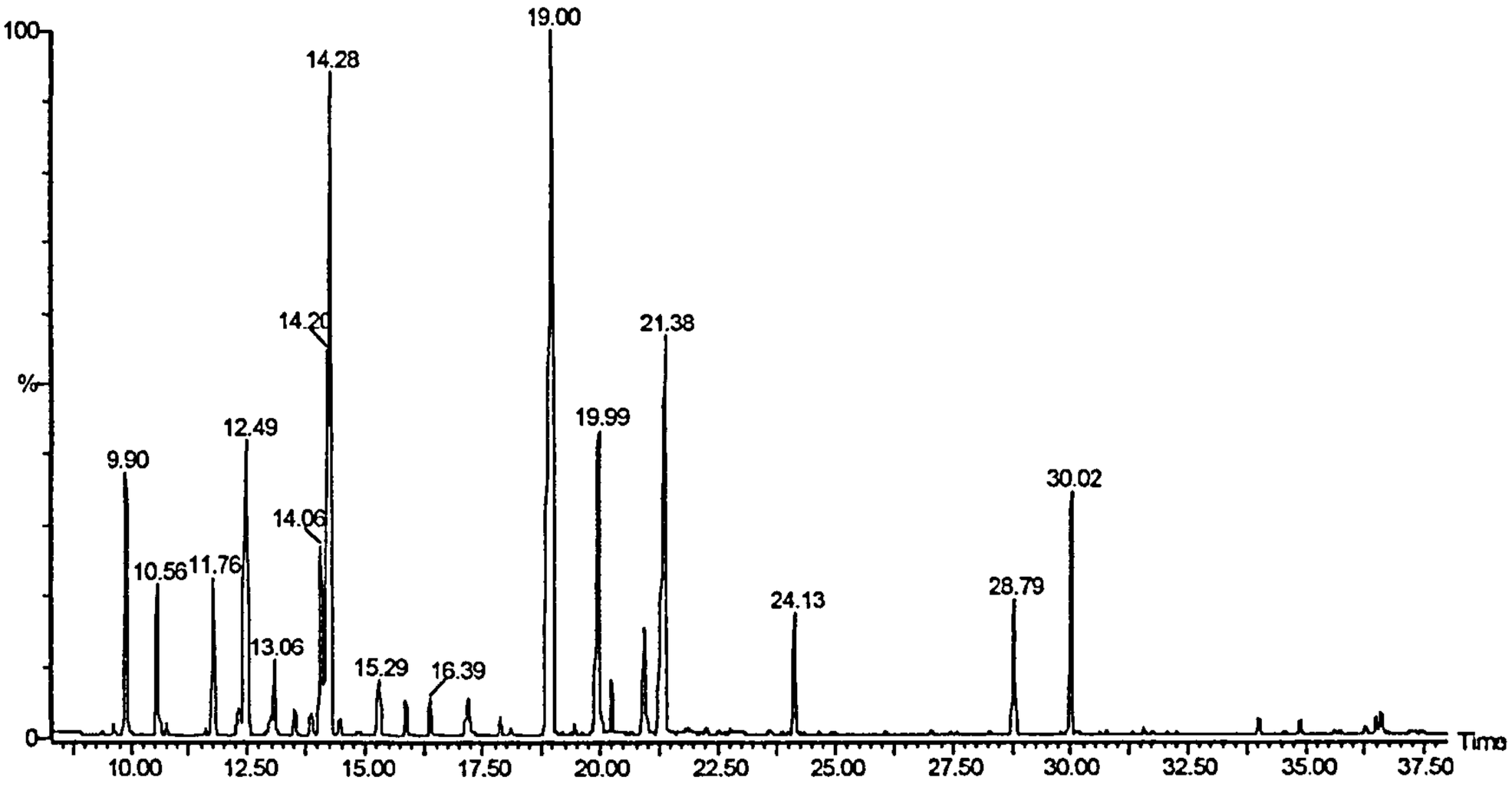


Figure A7. Total ion chromatogram of *Rosmarinus officinalis* fresh leaf (frozen) using GC-MS analysis.

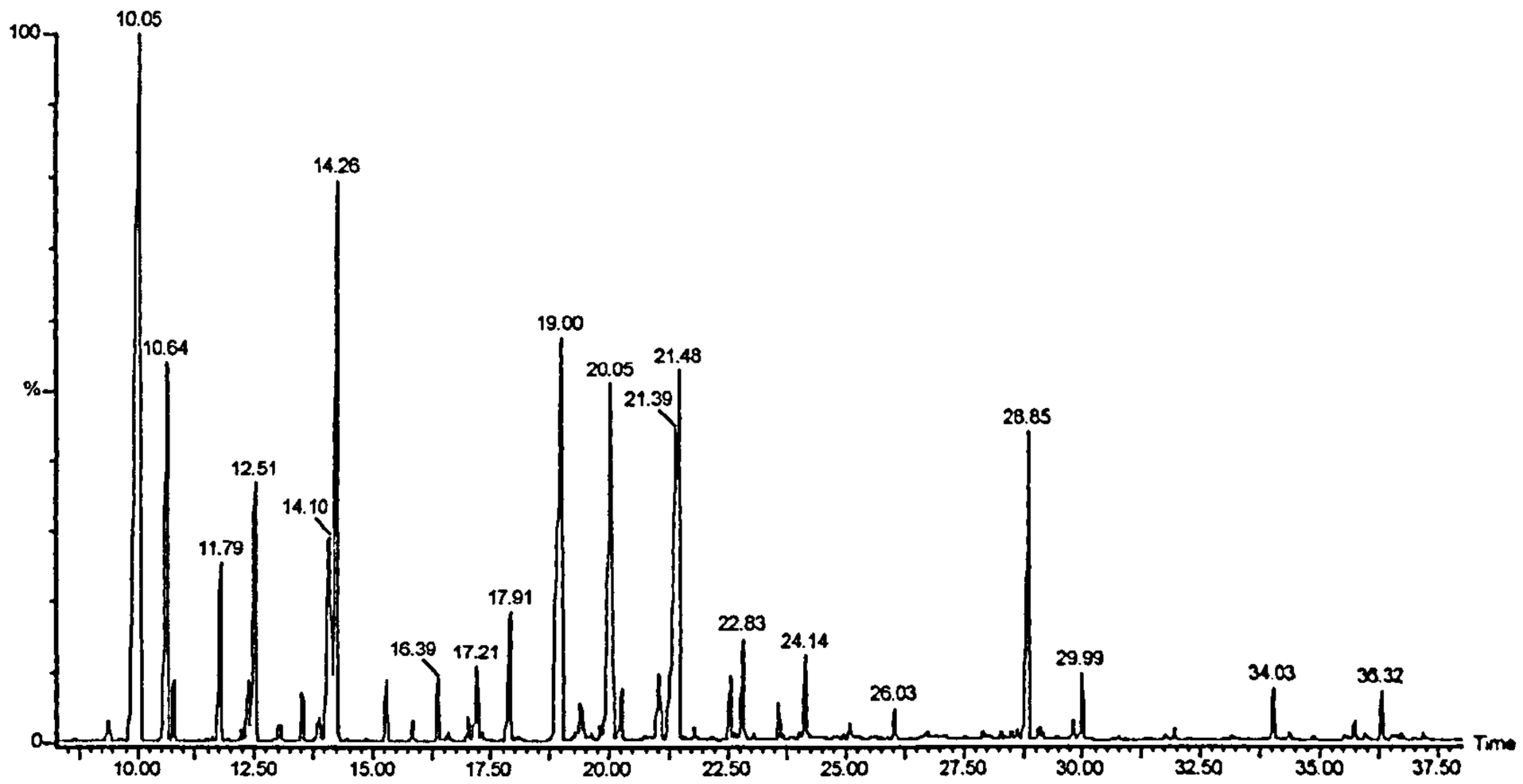


Figure A8. Total ion chromatogram of an authentic sample of *Rosmarinus officinalis* fresh leaf using GC-MS analysis.

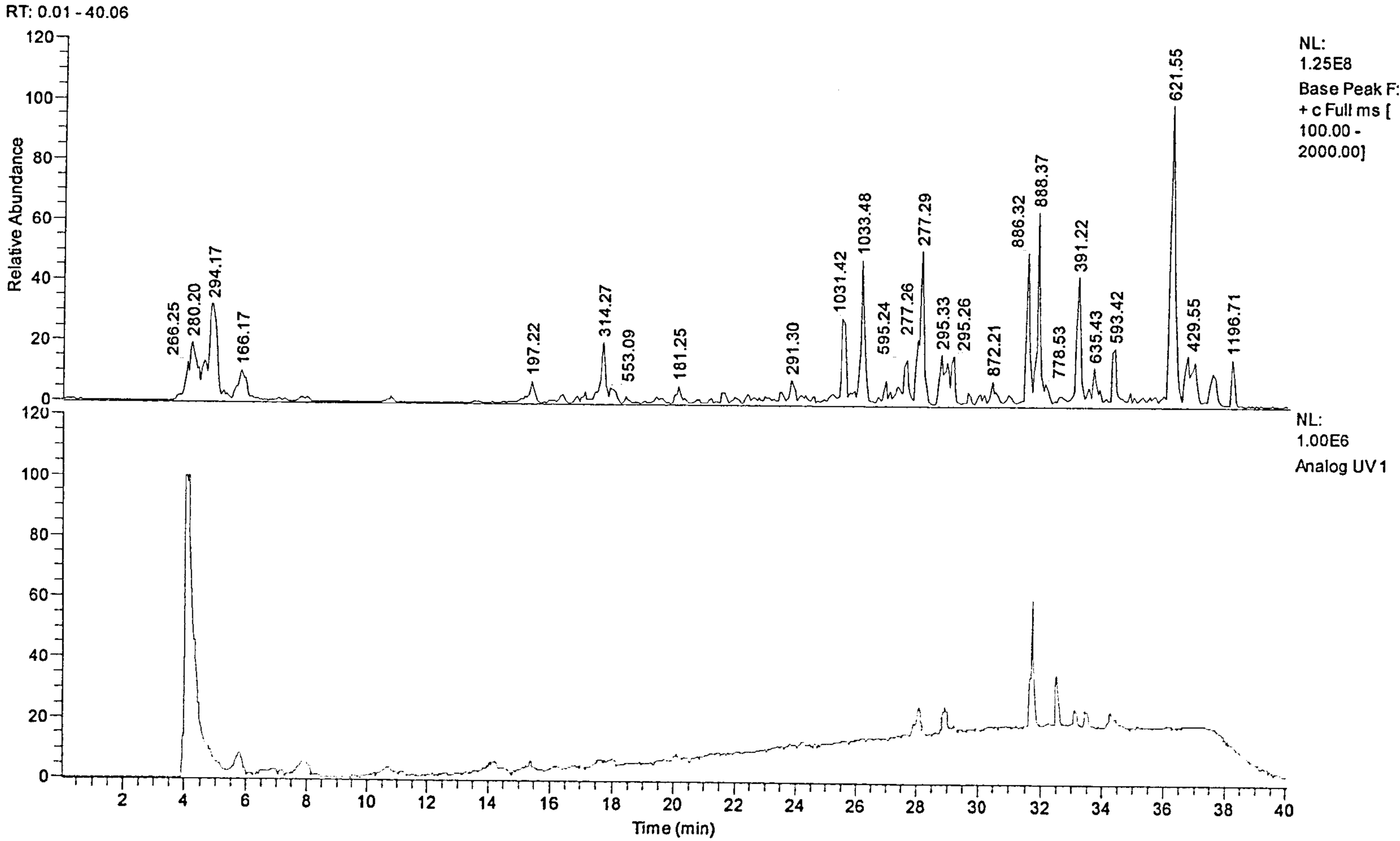


Figure A9. Chromatogram of *Convallaria majalis* leaf ethanol extract using LC-MS analysis and UV detection (210nm).

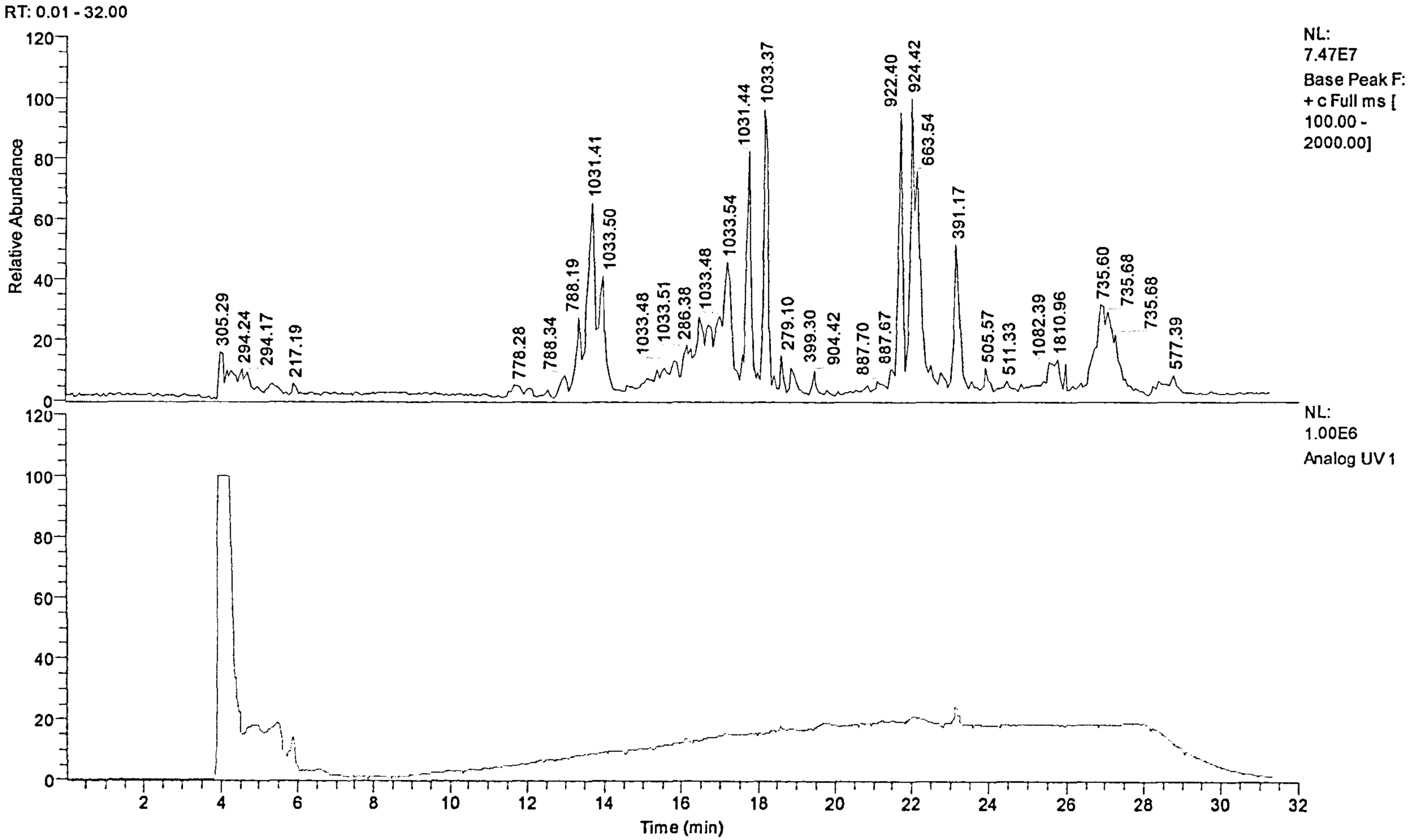


Figure A10. Chromatogram of preparative TLC fraction 1, from *Convallaria majalis* leaf ethanol extract, using LC-MS analysis and UV detection (210nm).

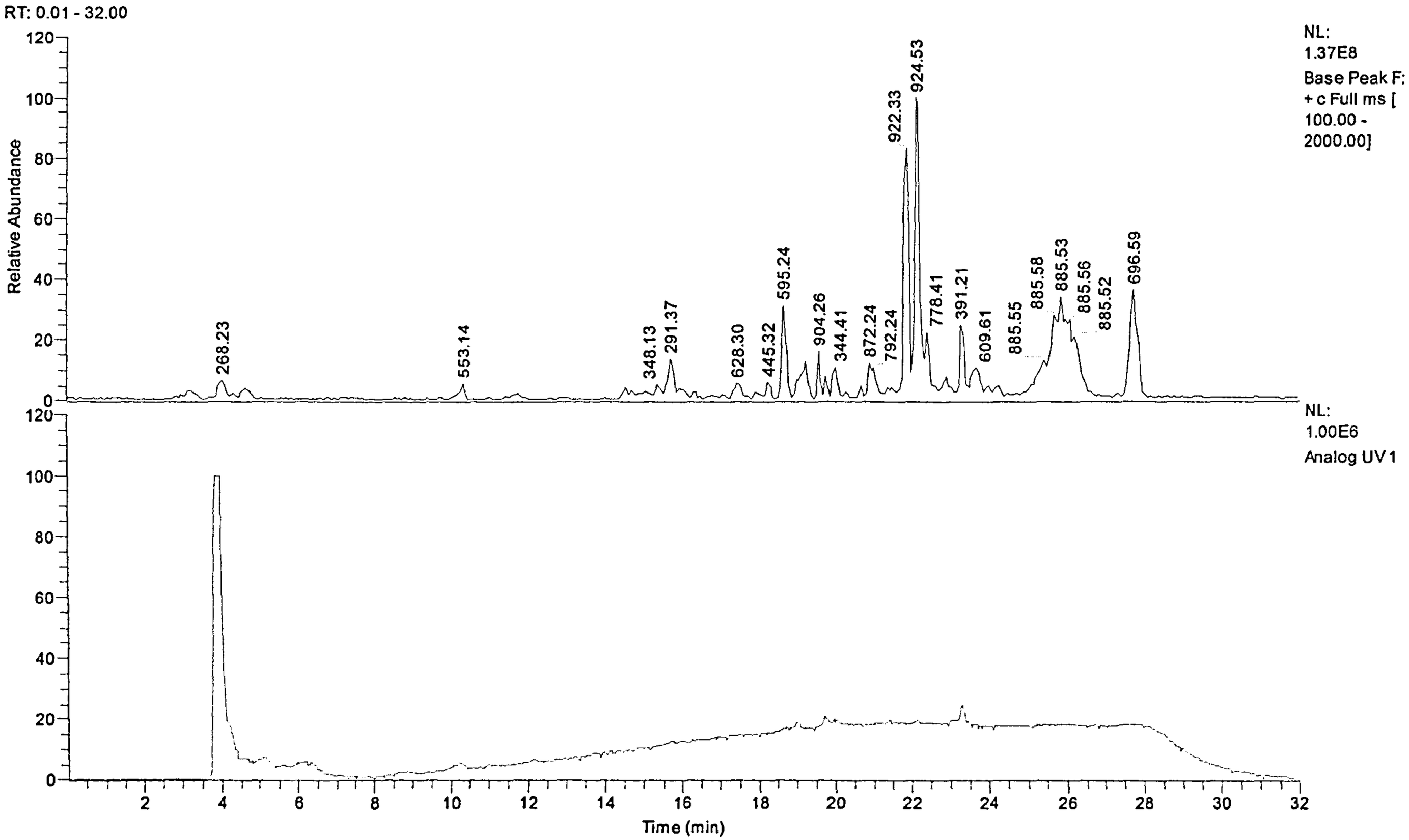


Figure A11. Chromatogram of preparative TLC fraction 2, from *Convallaria majalis* leaf ethanol extract, using LC-MS analysis and UV detection (210nm).

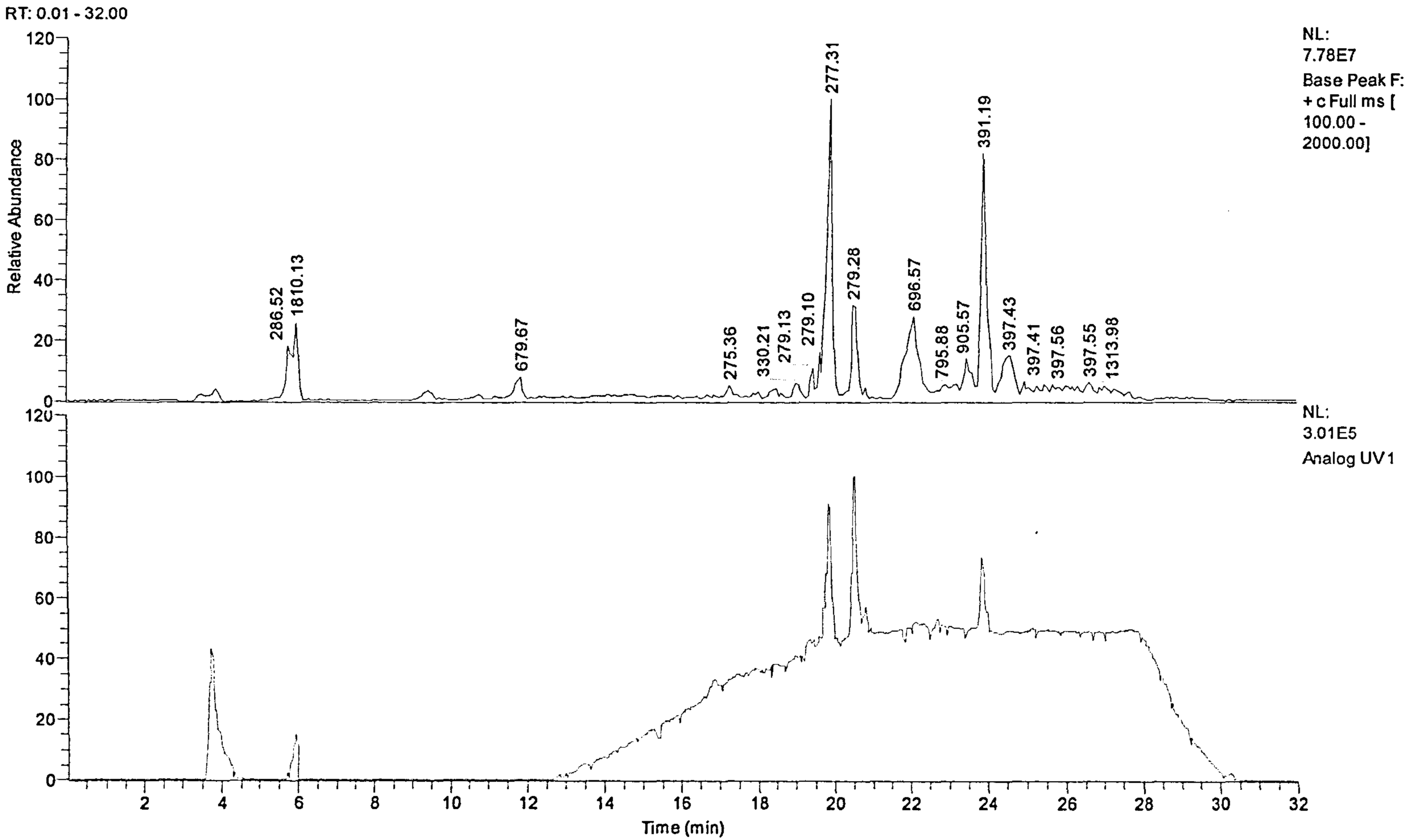


Figure A12. Chromatogram of preparative TLC fraction 3, from *Convallaria majalis* leaf ethanol extract, using LC-MS analysis and UV detection (210nm).

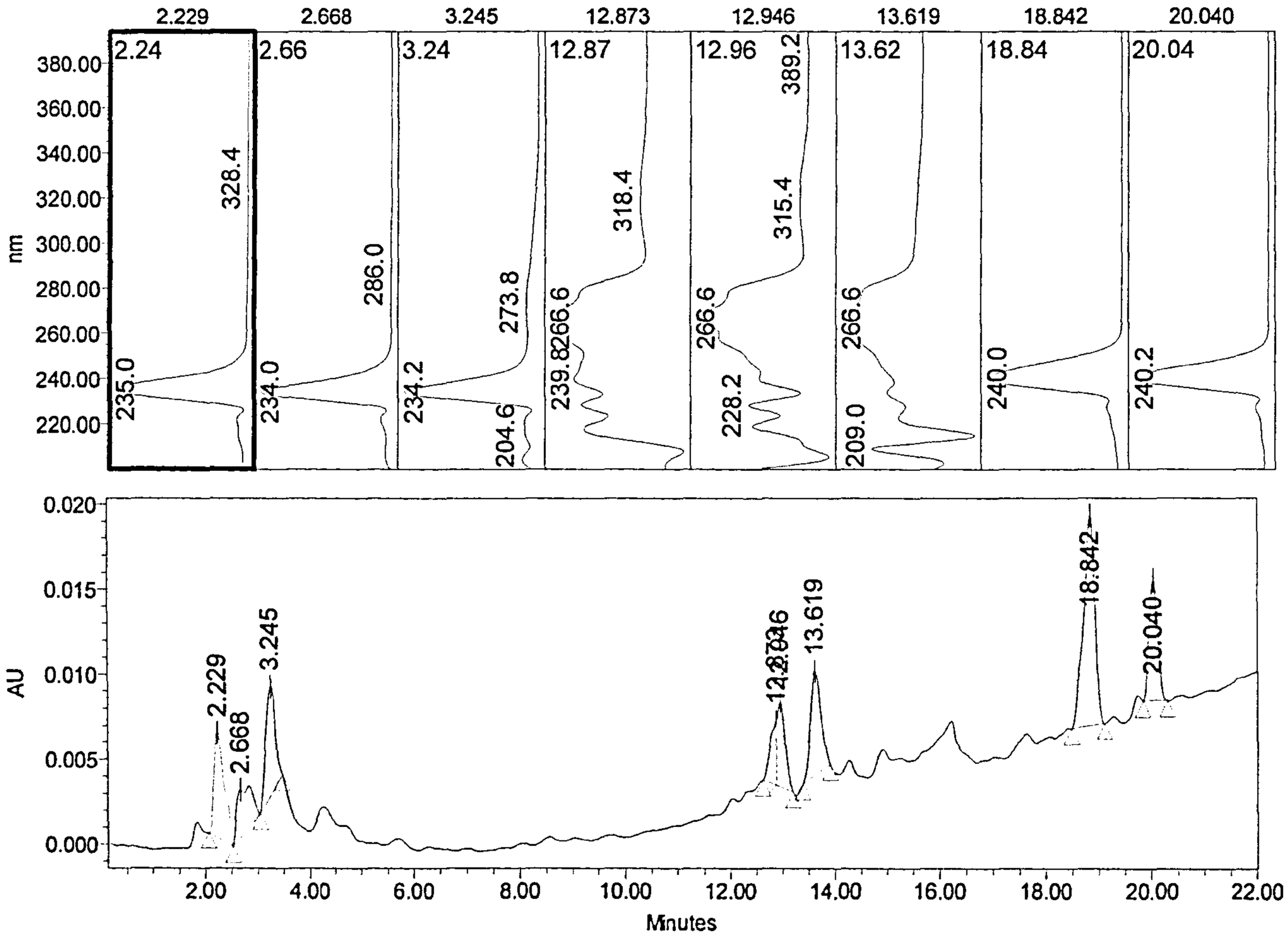


Figure A13. Chromatogram of preparative TLC fraction 3, from *Convallaria majalis* leaf ethanol extract, using HPLC analysis and UV detection (257nm).

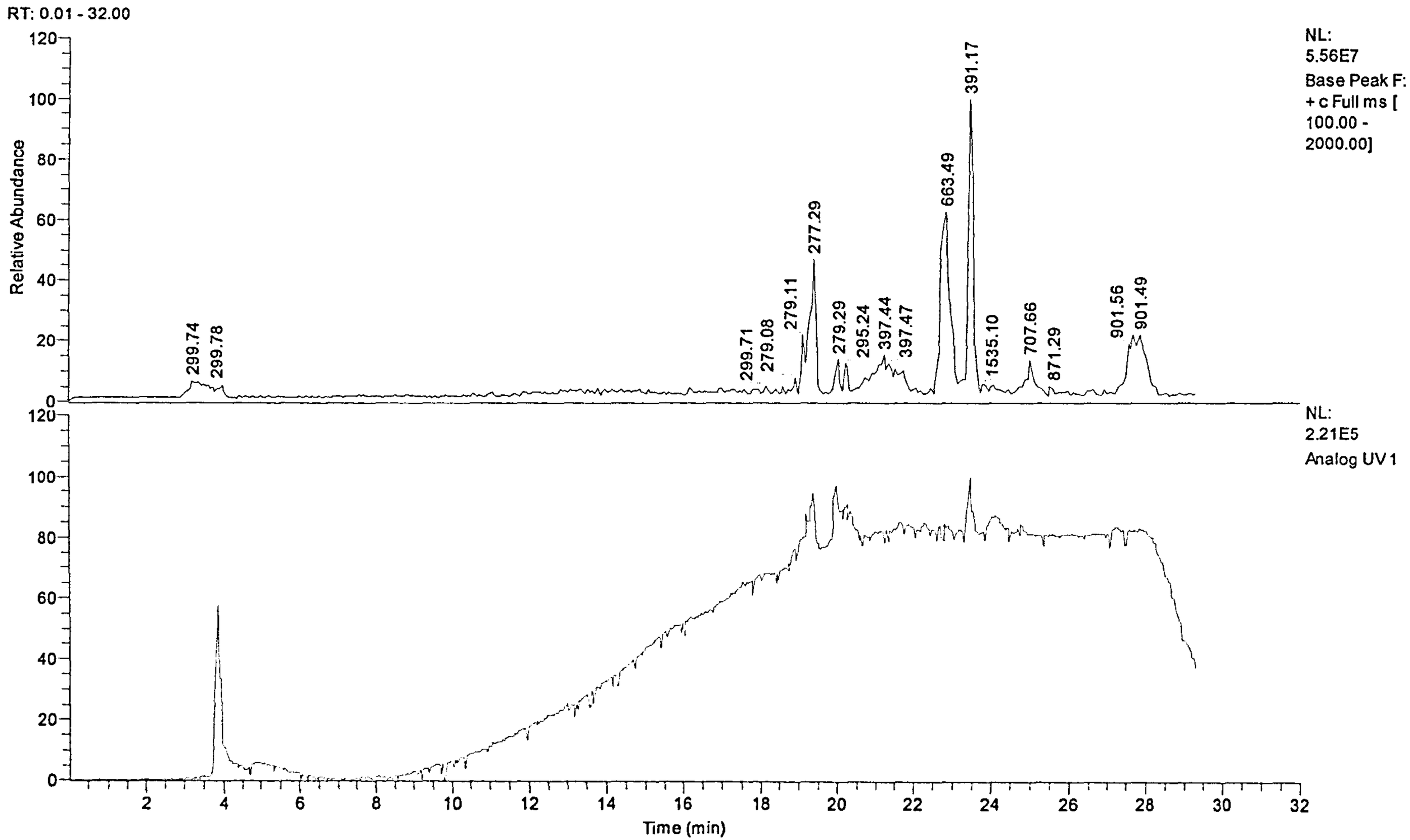


Figure A14. Chromatogram of preparative TLC fraction 4, from *Convallaria majalis* leaf ethanol extract, using LC-MS analysis and UV detection (210nm).

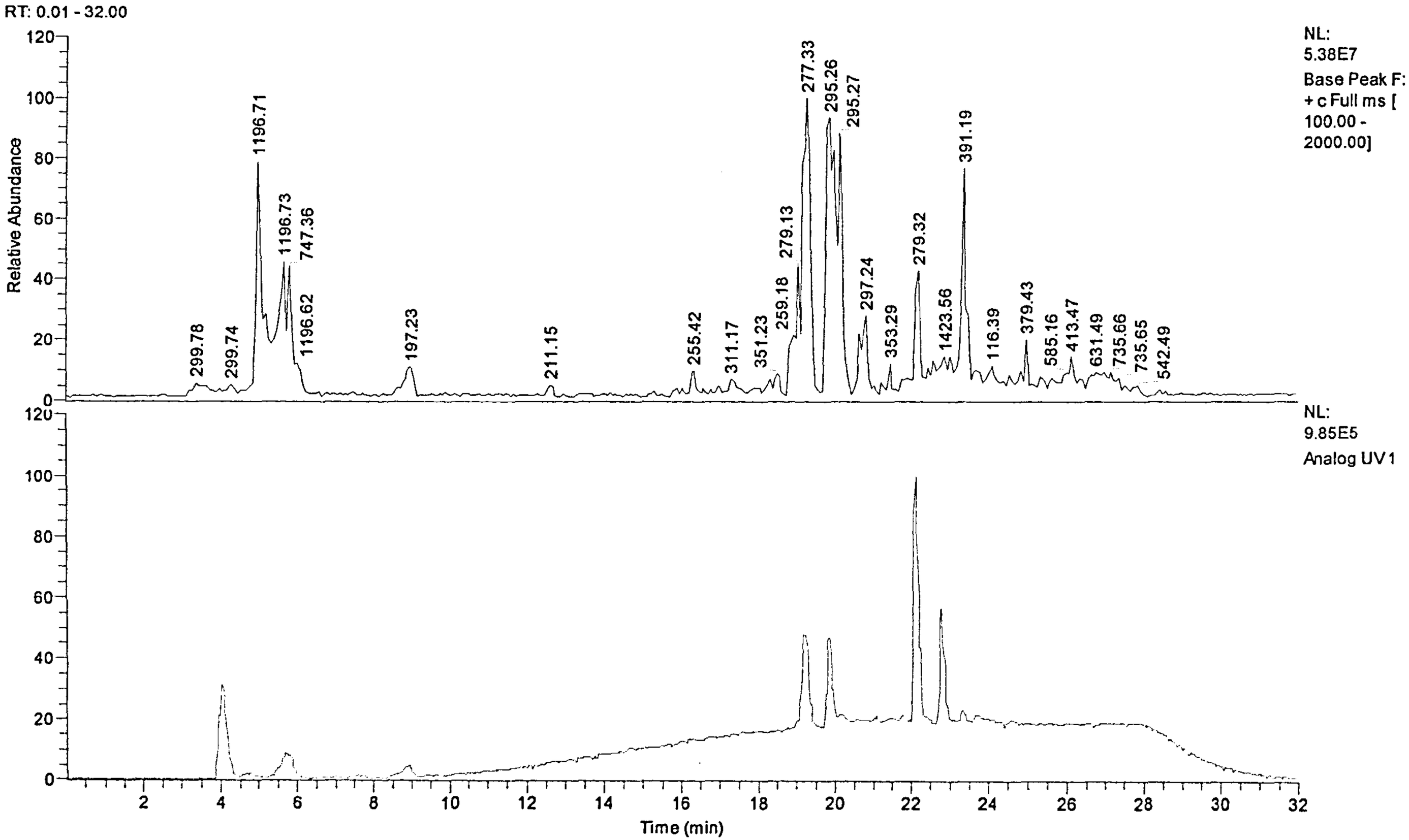


Figure A15. Chromatogram of preparative TLC fraction 5, from *Convallaria majalis* leaf ethanol extract, using LC-MS analysis and UV detection (210nm).

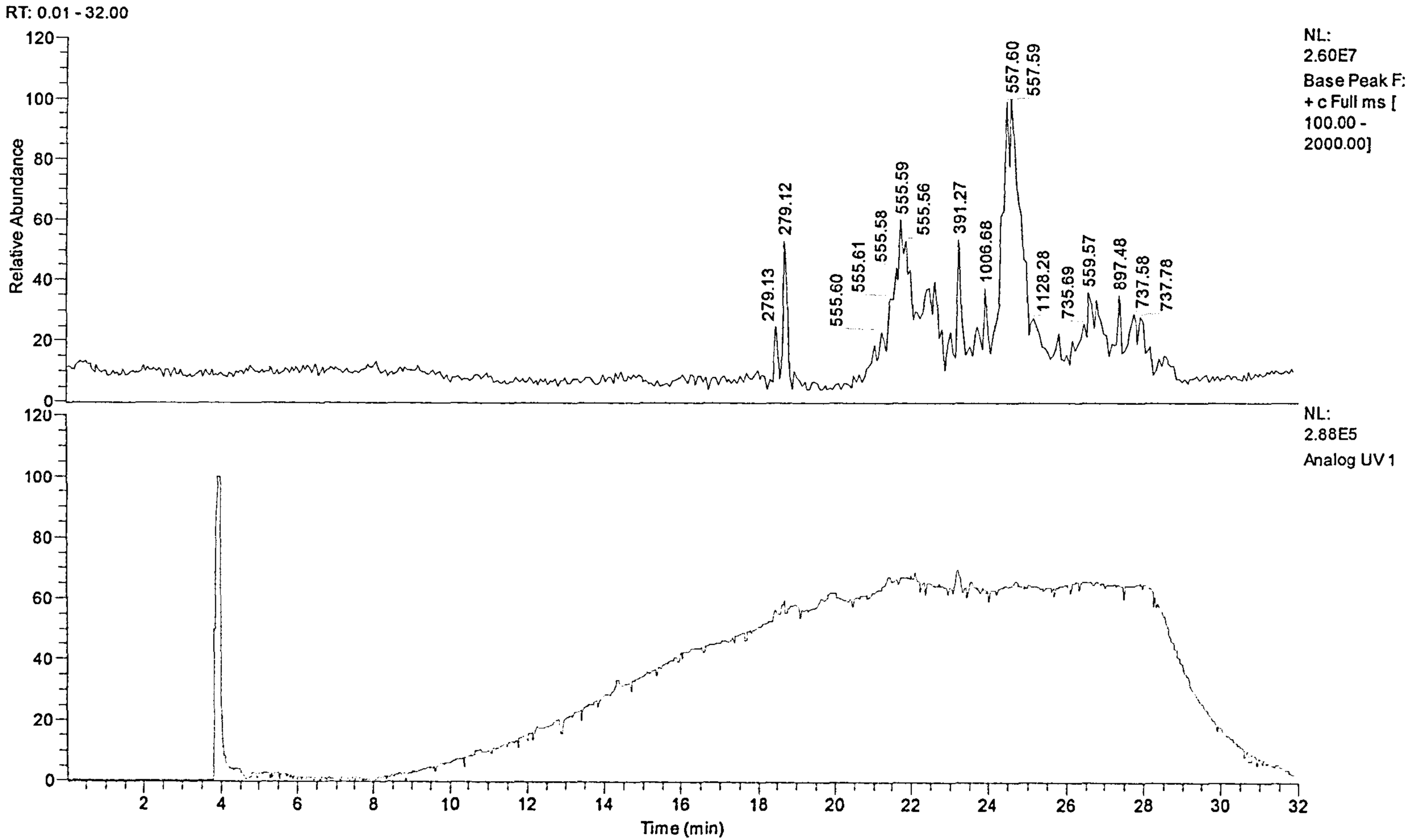


Figure A16. Chromatogram of preparative TLC fraction 6, from *Convallaria majalis* leaf ethanol extract, using LC-MS analysis and UV detection (210nm).

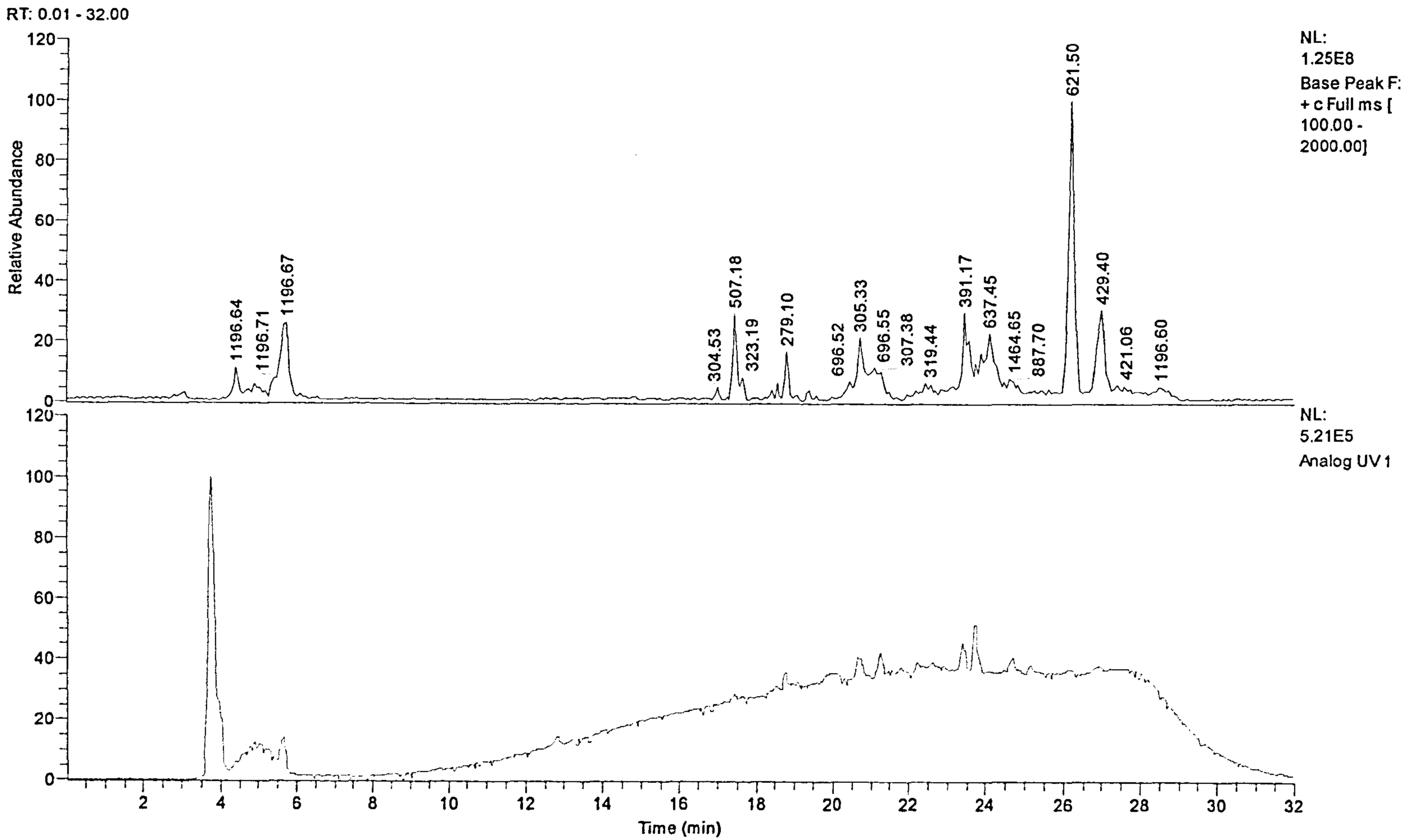


Figure A17. Chromatogram of preparative TLC fraction 7, from *Convallaria majalis* leaf ethanol extract, using LC-MS analysis and UV detection (210nm).

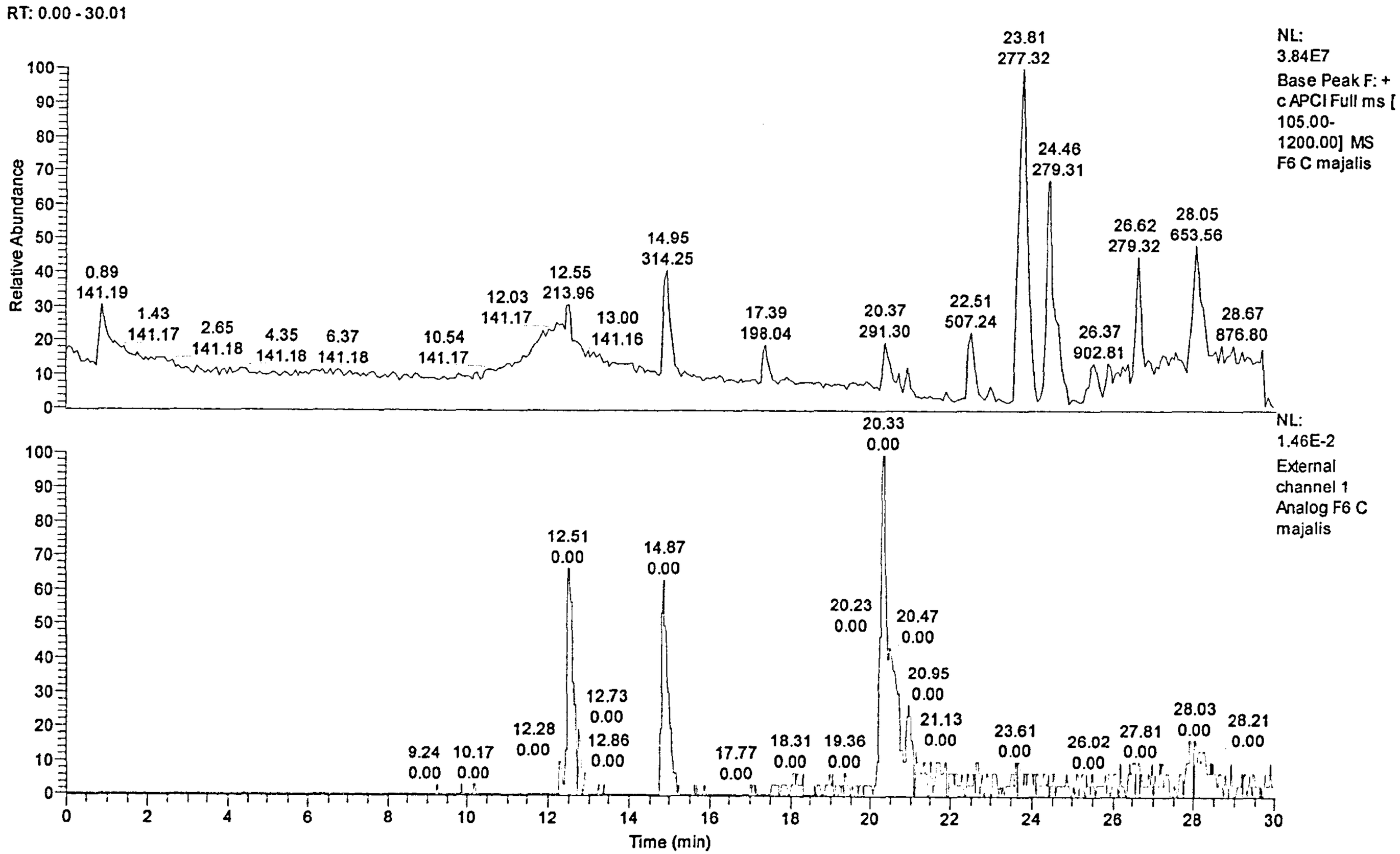


Figure A18. Chromatogram of flash column chromatography (FCC (b)) fraction 6, from *Convallaria majalis* leaf extract, using LC-MS analysis and UV detection (335nm).

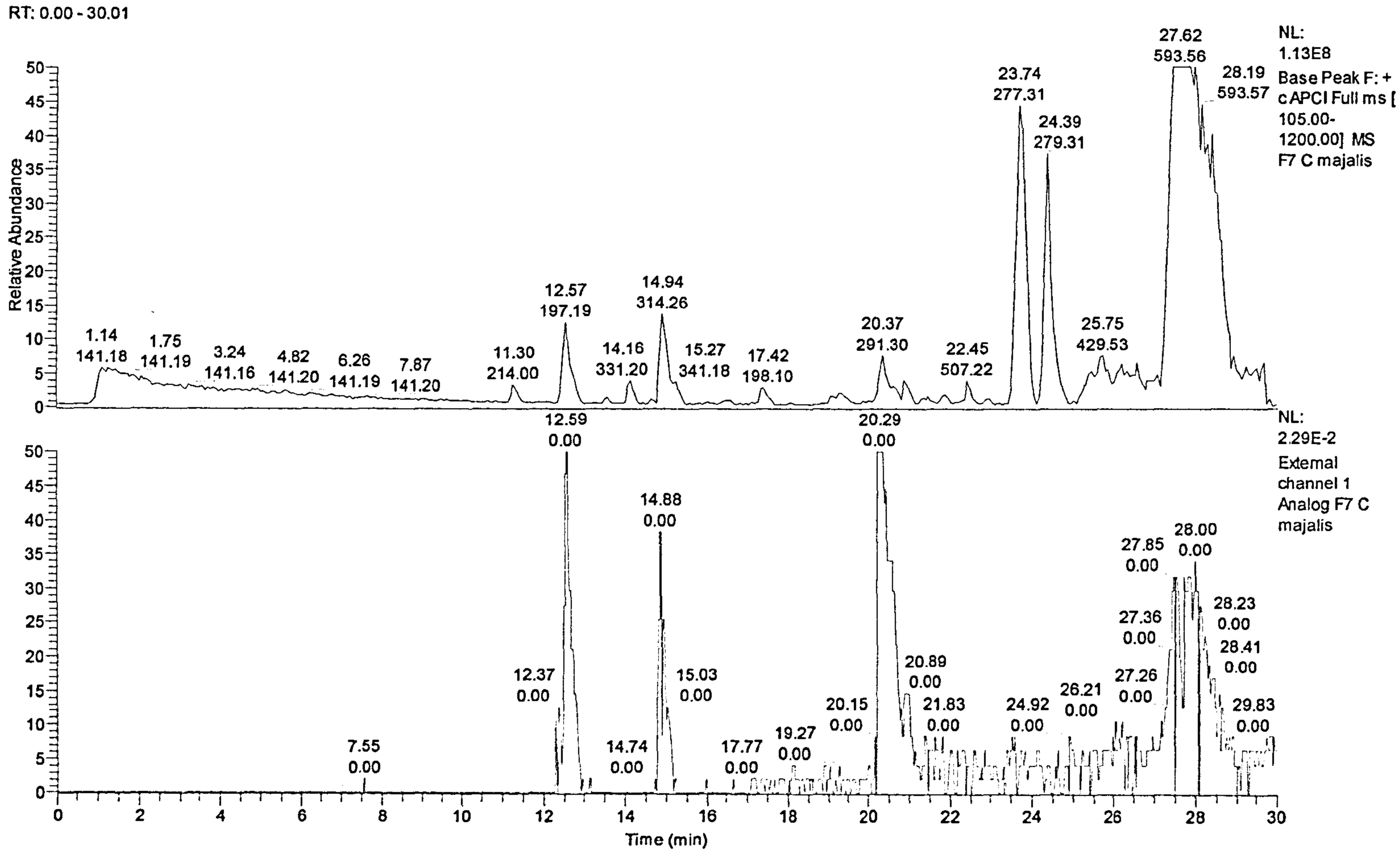


Figure A19. Chromatogram of flash column chromatography (FCC (b)) fraction 7, from *Convallaria majalis* leaf extract, using LC-MS analysis and UV detection (335nm).

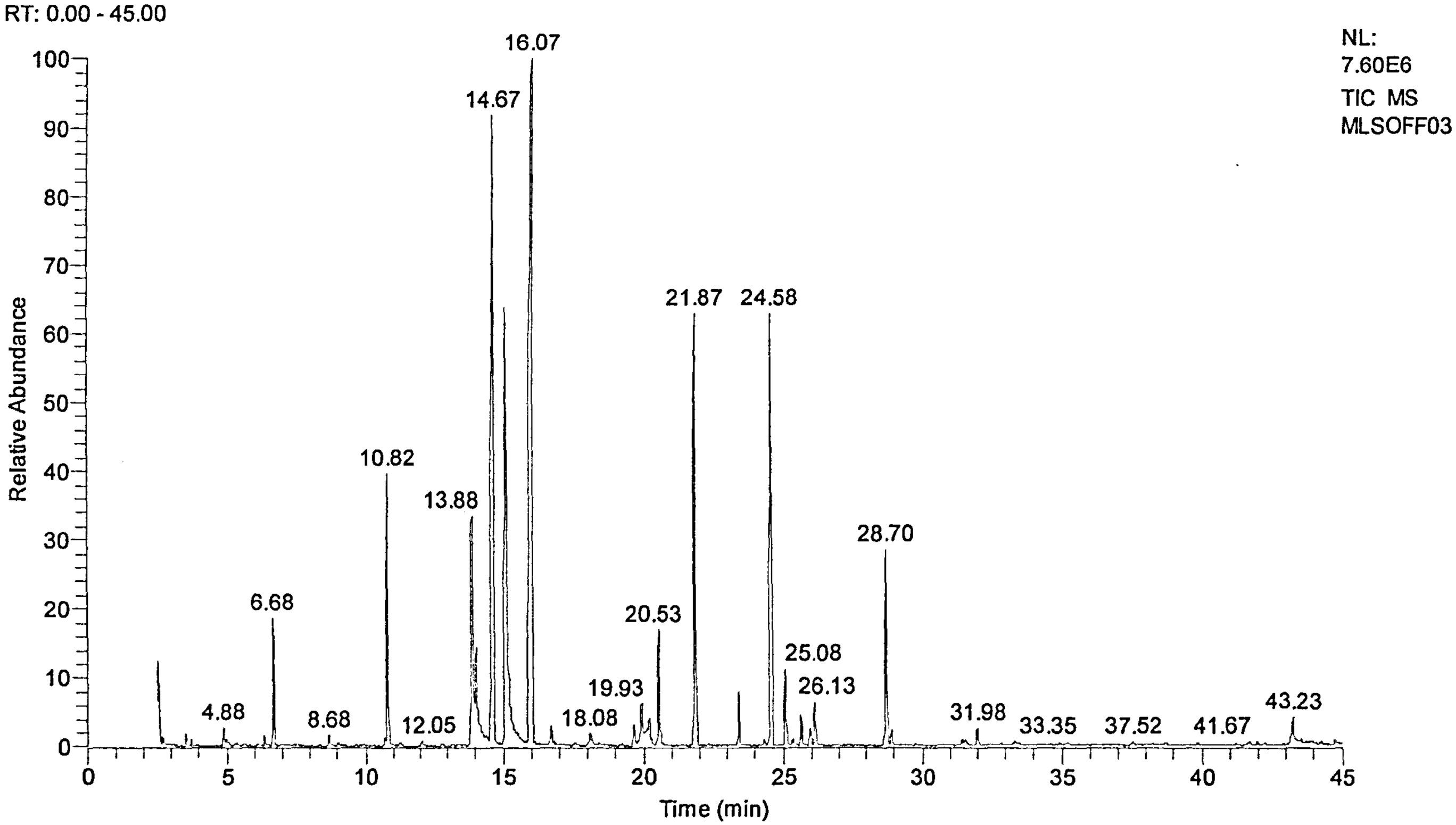


Figure A20. Total ion chromatogram of *Melissa officinalis* phytol extract (Clwydian Fragrant Oils) using GC-MS analysis.

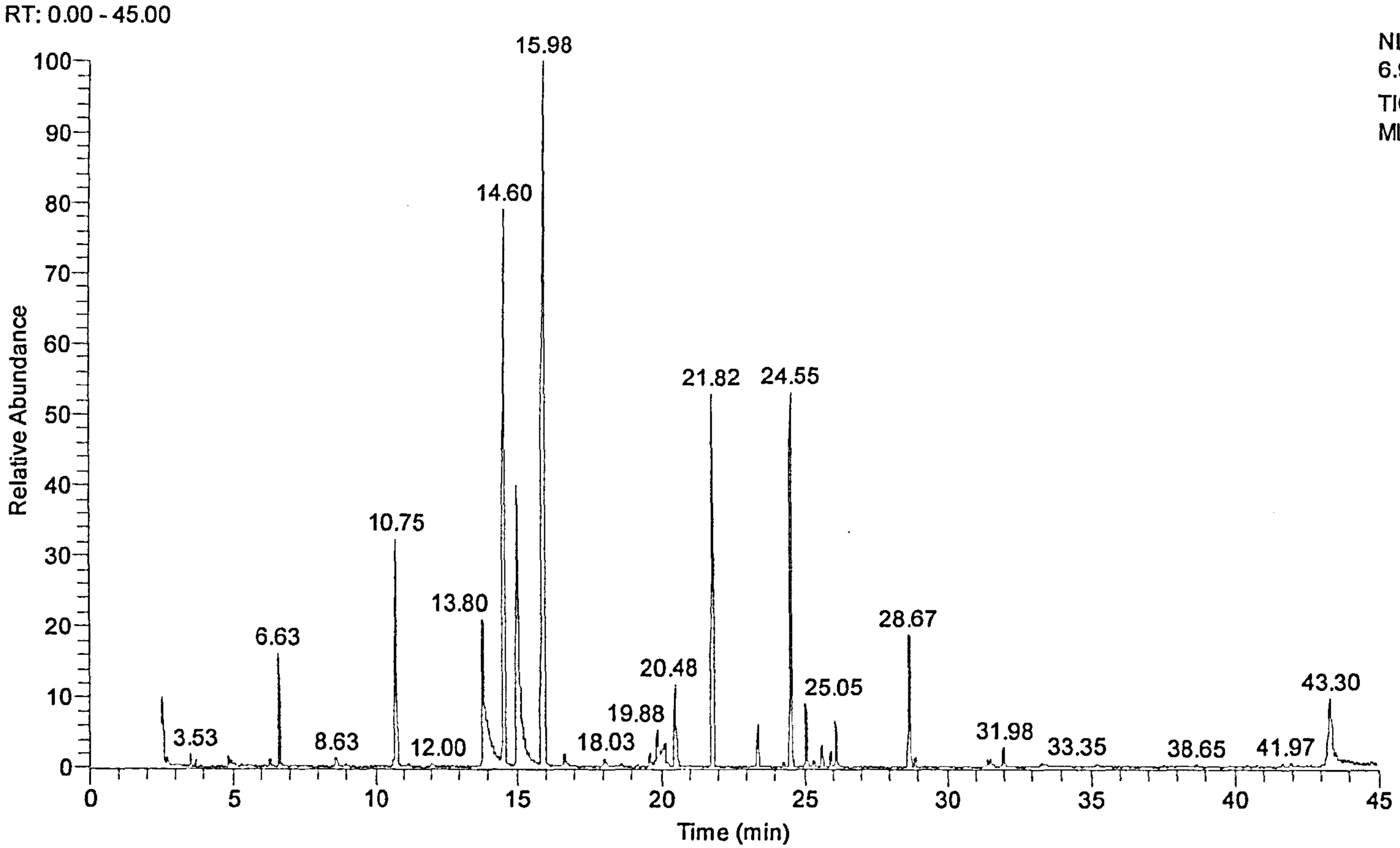


Figure A21. Total ion chromatogram of *Melissa officinalis* phytol extract (crude) (Clwydian Fragrant Oils) using GC-MS analysis.

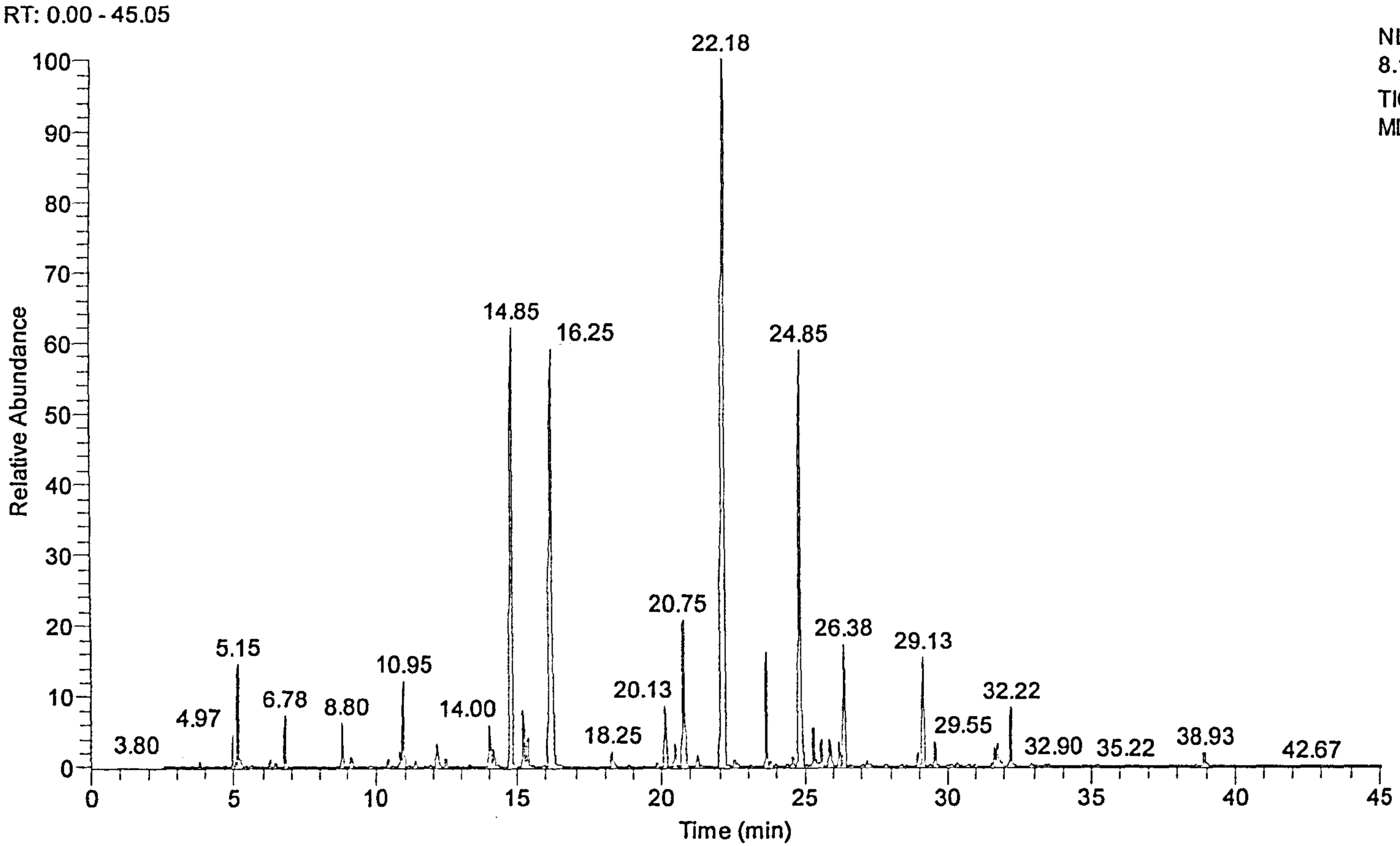


Figure A22. Total ion chromatogram of *Melissa officinalis* essential oil (Fragrant Earth) using GC-MS analysis.

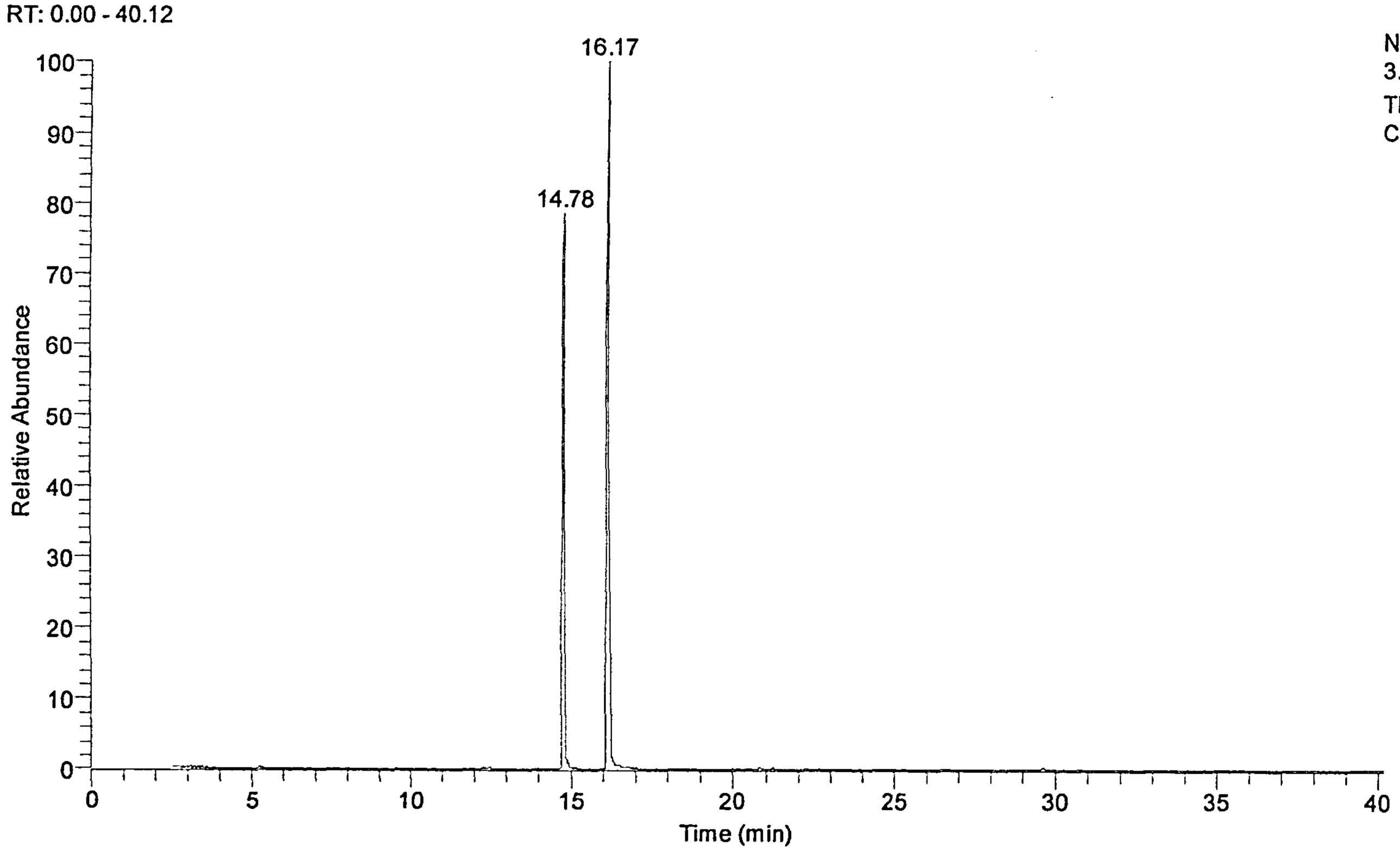


Figure A23. Total ion chromatogram of citral (Aldrich) using GC-MS analysis.

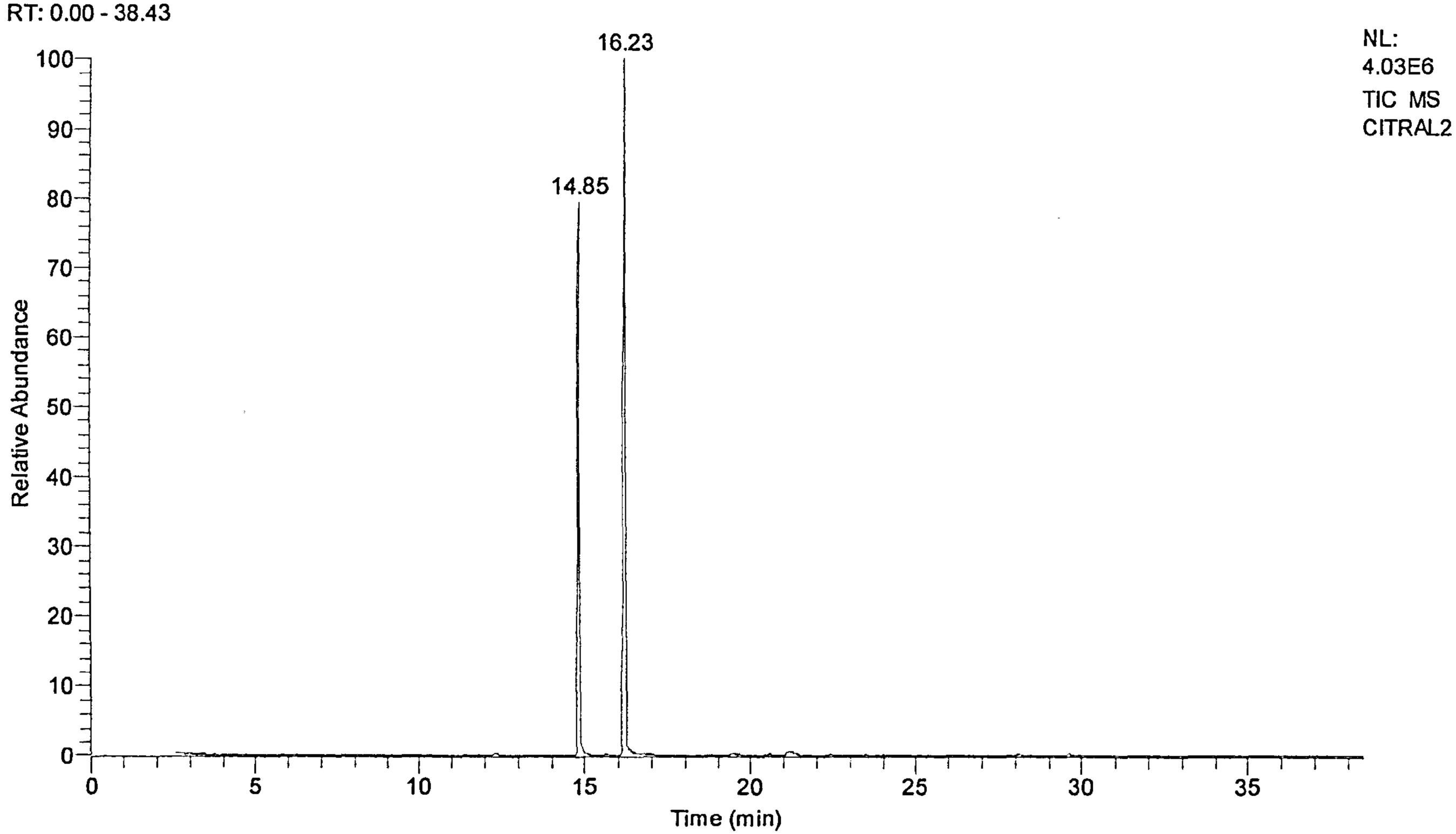


Figure A24. Total ion chromatogram of citral (Lancaster) using GC-MS analysis.

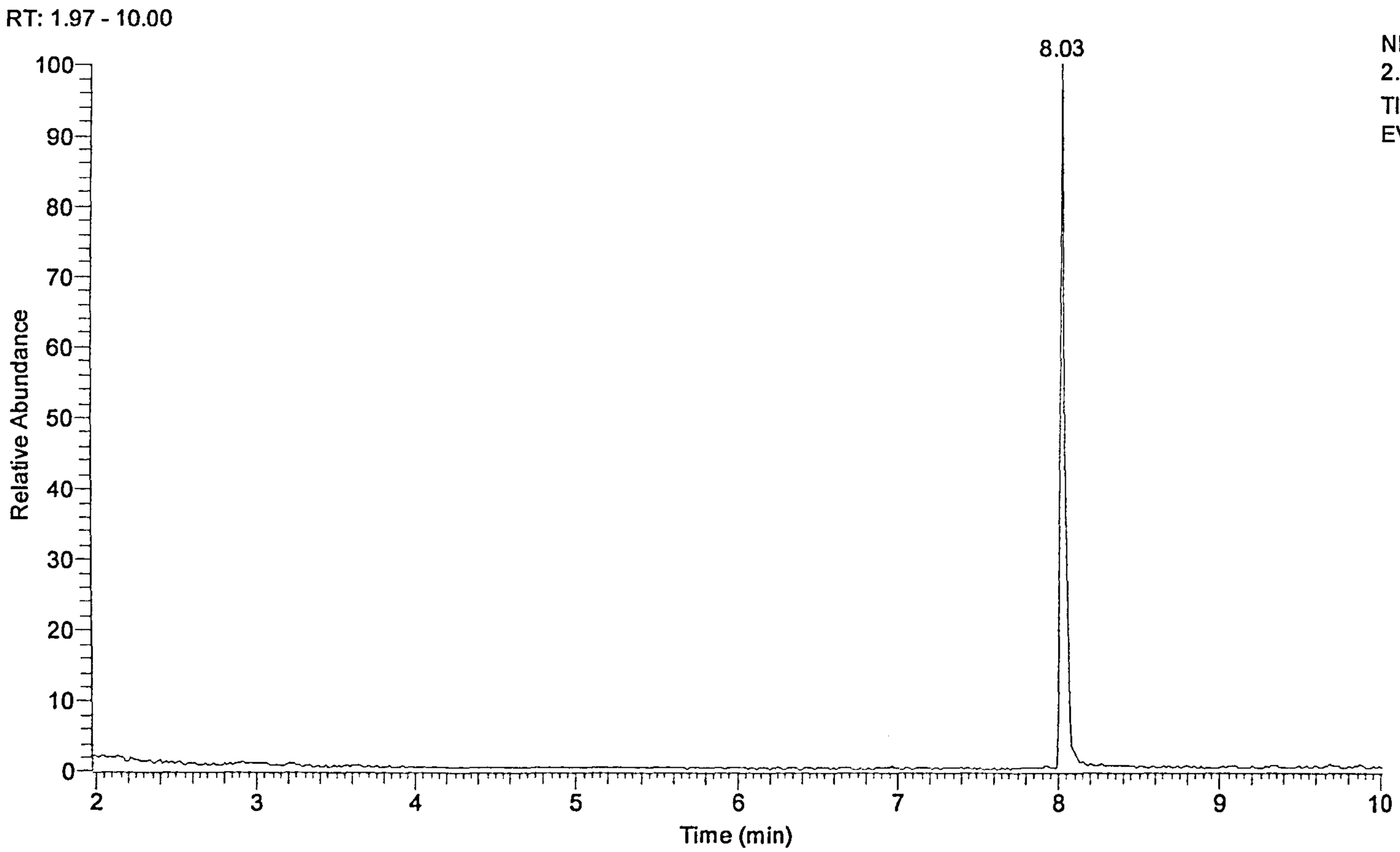


Figure A25. Total ion chromatogram of eugenol (Aldrich) using GC-MS analysis.

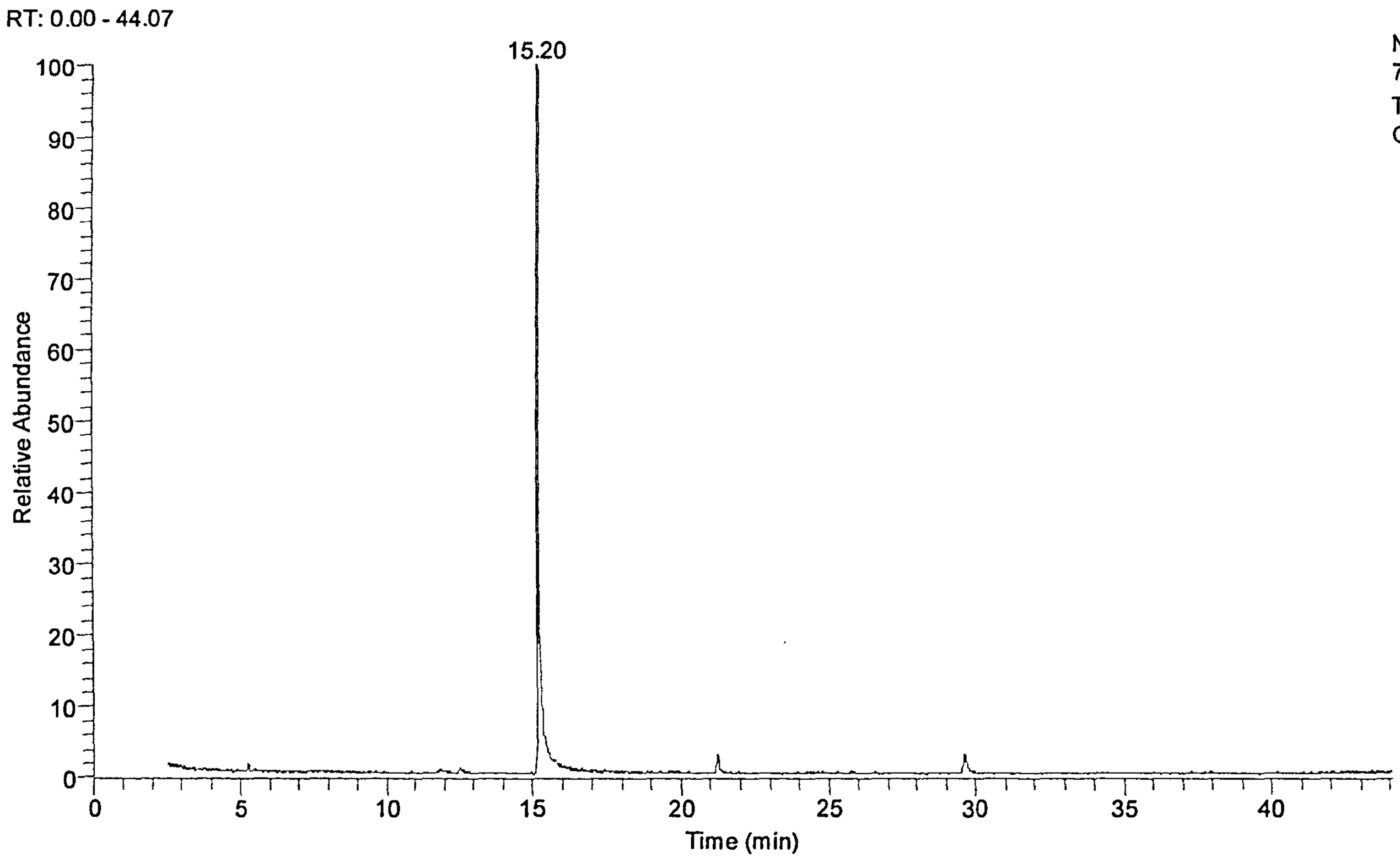


Figure A26. Total ion chromatogram of geraniol (Sigma) using GC-MS analysis.

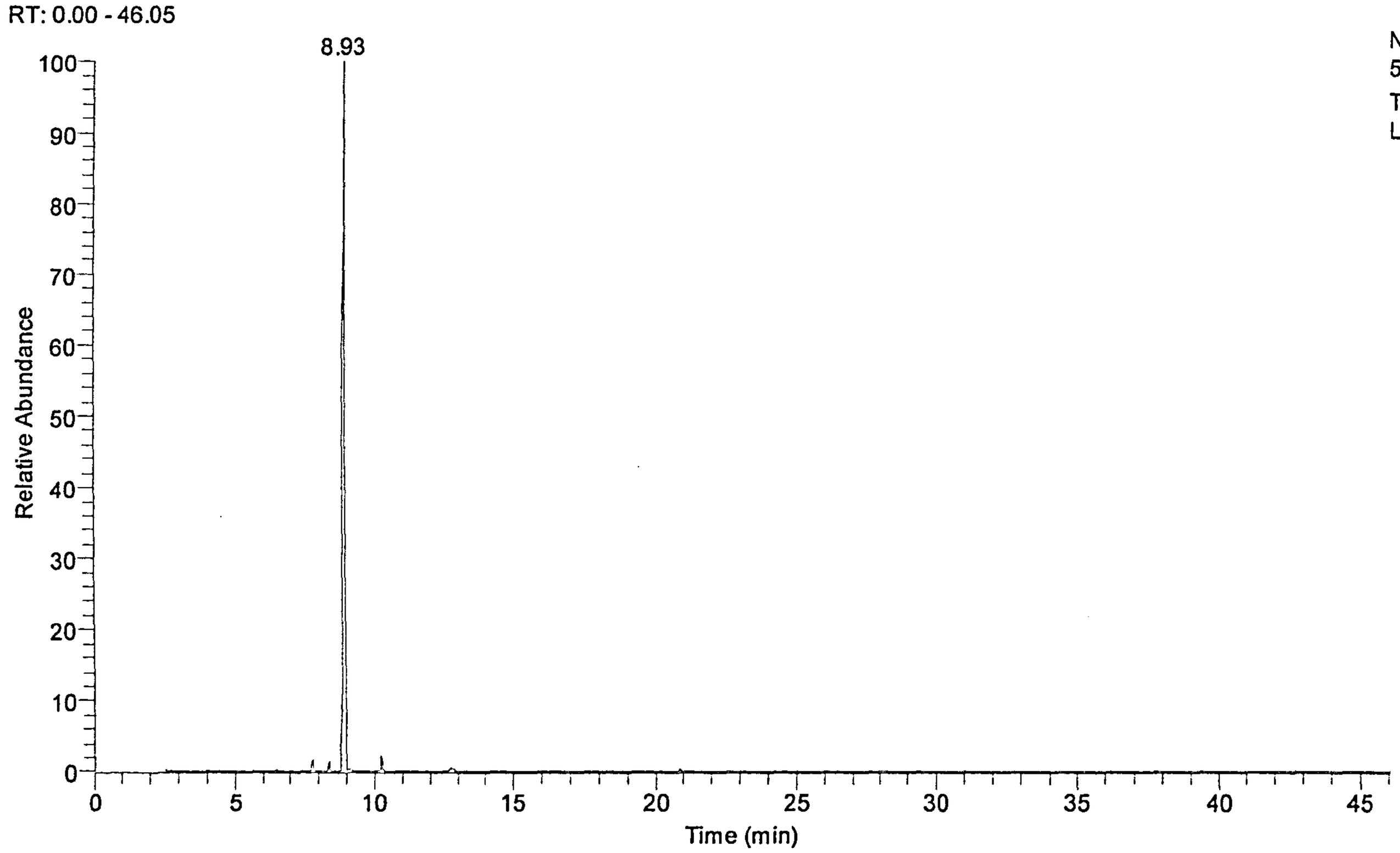


Figure A27. Total ion chromatogram of linalool (Aldrich) using GC-MS analysis.

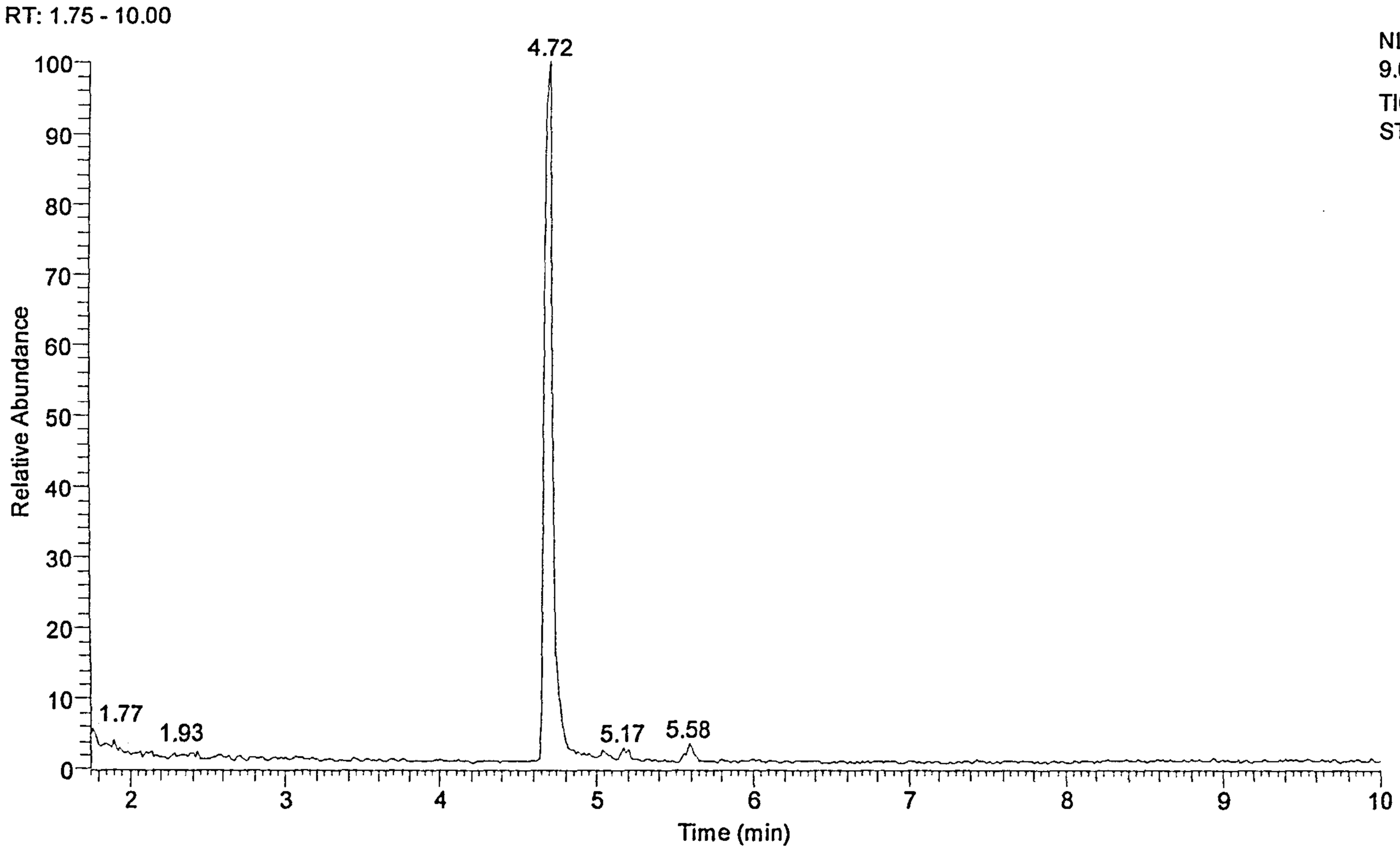


Figure A28. Total ion chromatogram of nerol (Sigma) using GC-MS analysis.

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